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TITLE: The Mechanosensory Ca²⁺ Channel as a Central Regulator of Prostate Tumor Cell Migration and Invasiveness

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14. ABSTRACT Our patch clamp studies indicate MscCa is expressed by the invasive prostate tumor cell PC-3. Anti-MscCa agents, Gd3+, GsmTx-4, and an anti-TRPC1 antibody block PC-3 cell migration. MscCa activity can be recorded over the surface of the PC-3 cell but is expressed at higher density on the rear compared with the front of the cell. This channel density gradient combined with a higher density of thapsigargin-sensitive Ca2+ stores in the rear of the cell enables the development of an intracellular Ca2+ gradient (low front –high rear) in migrating PC-3 cells that determines migration directionality. Gene silencing of TRPC1 and/or TRPC3, but not TRPC4 or TRPC6, blocks PC-3 cell migration. Permanently suppressing TRPC1 also reduces PC-3 cell proliferation and thereby blocks tumor invasion in vivo. The non-invasive human prostate tumor cell line LNCaP expresses MscCa but the channel undergoes rapid inactivation that prevents Ca2+ gradient development and directional cell migration. Our results indicate that specific forms of mechanical stimuli can switch the inactivating gating mode to the non-inactivating mode seen in PC-3 cells, and this switch is independent of the actin-cytoskeleton. These findings have specific implications regarding the possible role of the increases mechanical forces (e.g., solid stress and interstitial fluid compression) that develop within a growing prostate tumor in promoting its progression to malignancy.					
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Introduction

A major challenge for treating prostate cancer (PC) is to discover new therapies that will prevent the spread of PC cells from the prostate to distal sites. Our research focuses on the mechanosensitive Ca^{2+} permeant channel (MscCa), as a central regulator of prostate tumor cell migration. Our experiments are designed to address the two most basic issues of the disease: the mechanism(s) that trigger progression of PC to malignancy and the urgent need for new therapeutic targets to block or reverse this progression. Our original experiments funded by DOD were aimed to test whether MscCa is expressed in human prostate tumor cells and whether MscCa activity is required for prostate tumor cell migration. We confirmed both results. In the course of these experiments we also discovered that the predominate gating mode of the MscCa differs between noninvasive and invasive PC cells, and this is the most powerful determinate of the $[\text{Ca}^{2+}]_i$ dynamics required to coordinate cell locomotion. The aims of the current award were three-fold. First, determine the mechanisms underlying MscCa gating. Second, determine the cancer-related processes that switch MscCa gating, and third determine whether anti-MscCa conditions that suppress PC migration *in vitro* also block PC cell invasion *in vivo*. Insights into these aspects would provide added motivation for developing more selective therapies that target MscCa and its regulatory mechanisms. The additional relevance of the project was that we would extend our *in vitro* discoveries to the next preclinical level and test the ability of anti-MscCa treatments to block metastases *in vivo*.

We have completed the original tasks outlined in year 1, and the results of these studies have been described in a manuscript (see Maroto et al., 2008 and abstracts included as Appendix I). Also as a result of experiments in year 1 our goals have been expanded to test the role of TRPC3 as well as TRPC1 in prostate tumor cell migration. In particular, our experimental results indicate that TRPC1 and TRPC3 (but not TRPC4, 6 or 7) are required for prostate tumor cell migration. Part of our task of year 2 will be to determine whether TRPC1 and TRPC3 operate together or independently to regulate prostate tumor cell migration. Our results indicate that TRPC1 is a subunit of MscCa (Maroto et al., 2005). However, new experiments in a collaborative effort (Gottlieb et al., 2008) indicate that when TRPC1 is over expressed it is not efficiently inserted in the cell membrane, but instead remains in the endoplasmic reticulum. This result indicates that additional protein subunits may be required for efficient channel insertion. Part of the task of year 2 will be to identify the subunits.

In year 1 we also began the *in vivo* studies, which were originally planned to commence in year 2. Our preliminary findings indicate that suppression of TRPC1, in addition to blocking tumor cell migration *in vitro* also suppresses tumor cell proliferation *in vivo*. This latter effect is most evident when the TRPC1-suppressed PC-3 tumor cells are implanted subcutaneously in nude mice. Unlike wild type PC-3 cells, these TRPC1-suppressed cells fail to grow sufficiently large enough tumors to enable harvesting for orthotopic implantation in the mouse prostate (i.e., in order to test for invasiveness). The dual effects of TRPC1 on PC-3 cell migration and cell proliferation are interesting but also represent a challenge in terms of dissecting the underlying effects and their relative importance *in vivo*. One of our strategies in year 2 will be study PC3 cell sublines that are recognized to proliferate at high rates *in vivo* and test whether TRPC1 suppression in these cells still allows for *in situ* tumor growth.

Based on our observation that the transient gating mode/inactivation of MscCa is mechanically fragile, we hypothesized that an intact actin-cytoskeleton (CSK) was required for inactivation, and that mechanical stimuli could disrupt the actin-CSK and thereby abolish channel inactivation. However, our experiments in year 1 with several specific agents that affect actin polymerization/depolymerization failed to alter MscCa gating. Based on our observation that MscCa is retained in pure liposomes (Maroto et al., 2005) we will test the role of the lipid bilayer structure on MscCa gating (e.g., see Task 1.4).

Body

Task 1: Determine the mechanism(s) that regulate MscCa gating, expression and surface distribution in PC cells that display different invasiveness and metastatic potential.

- 1.1 Use patch-clamp/pressure clamp techniques, confocal immunofluorescence, Westerns and surface biotinylation techniques to measure gating, surface distribution and expression, respectively, of MscCa/TRPC1 in PC-3 and LNCaP cell lines.

Time line: Year 1, months 1-6.

Milestone: Establish a baseline for studying the effects of various agents and treatments that may alter these properties as described in tasks 1.2 to 1.4.

Surface distribution of MscCa activity on human prostate tumor cells

PC-3 cells: Utilizing the high spatial resolution of patch clamp recording we have demonstrated that MscCa is expressed over the membrane surface of migrating PC-3 cells, with MscCa activity detectable on the front lamella, the cell body, and the rear tether of the cell. Figure 1 (see Maroto, et al., 2008, pdf version included in Appendix 1) shows a photomicrograph of a PC-3 cell and representative recordings made on the indicated cellular regions.

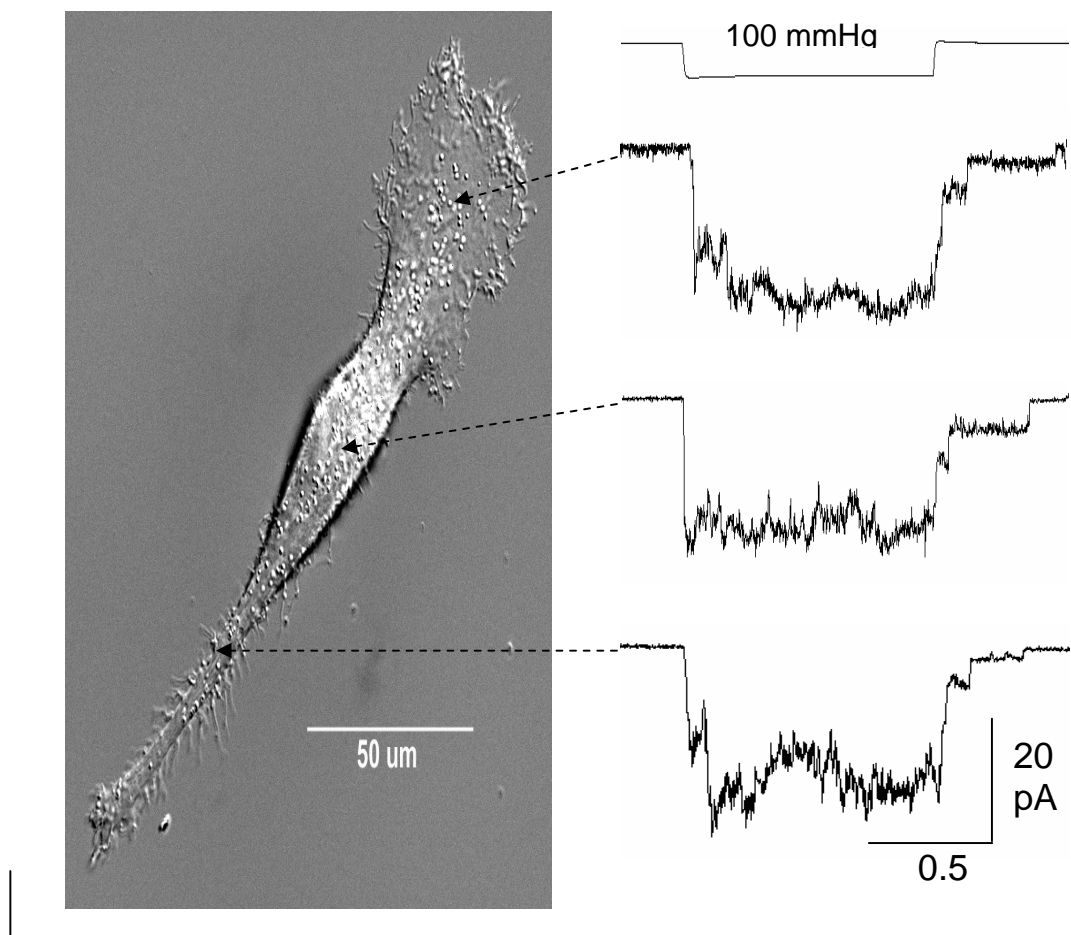


Fig. 1. The left panel is a photomicrograph of a PC-3 cell. The right panels show patch recordings made from the specified regions of PC-3 cells.

Quantitative analysis of patch recordings made on over 100 PC-3 cells indicate that MscCa is found on all regions of the PC-3 cell but it is expressed at a higher density on the rear compared with the front. In particular, we found the percentage of null vs active MscCa patches was higher on the lamella (L: 30%, 10 vs 32) compared with either the cell body (B: 18%, 8 vs 46) or the cell rear (R: 12%, 3 vs 25).

The above patch results **are significant** because they represent the first patch recordings made from any migrating tumor cell and the first mapping of the surface distribution of MscCa. Our findings are also important because they directly address a controversy on whether MscCa activity is restricted to the front or the rear of migrating cells. As described below the patch results can be directly compared with immunological studies in which we have mapped the distribution of surface Ca^{2+} channels and internal Ca^{2+} stores.

LNCaP cells: Our patch results on the LNCaP cells indicate that MscCa current density is around 10-fold higher on LNCaP cells compared with PC-3 cells and the activity is evenly distributed over the cell surface (Fig. 2, see also Maroto et al. 2008).

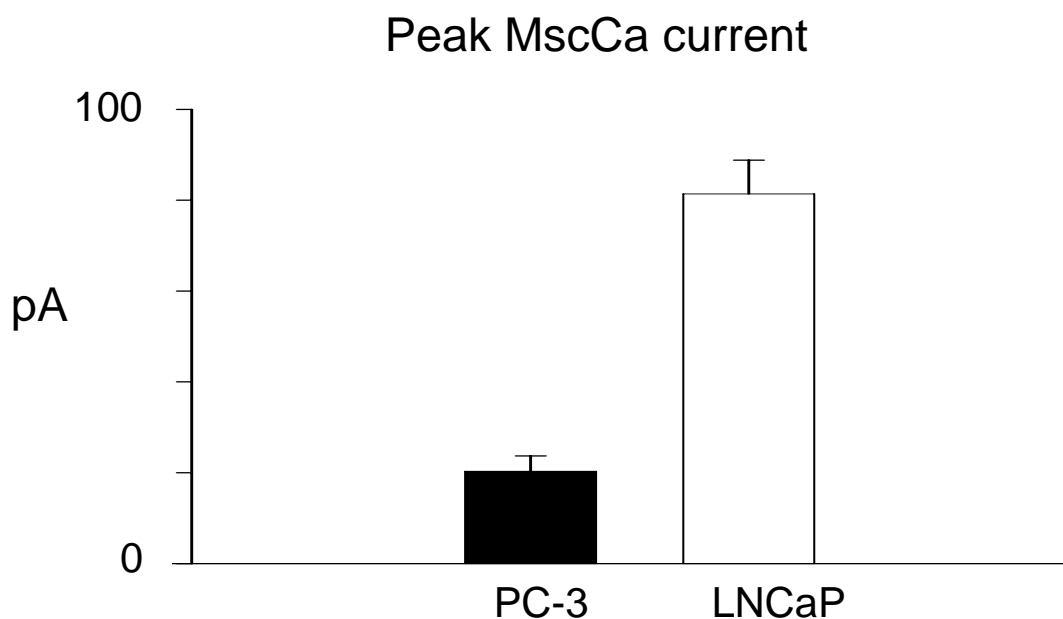


Fig.2 Histogram showing the average current density of MscCa measured on cell-attached patches formed on PC-3 and LNCaP cells.

The higher expression of MscCa on the non-migrating LNCaP cell compared with the migratory PC-3 cell was clear cut and not anticipated. However, as described below this result agrees with the higher expression of TRPC1 in LNCaP vs PC-3 cells as measured by Westerns and by immunofluorescence (see also Maroto et al., 2008).

Comparison of gating properties of MscCa in PC-3 and LNCaP cells

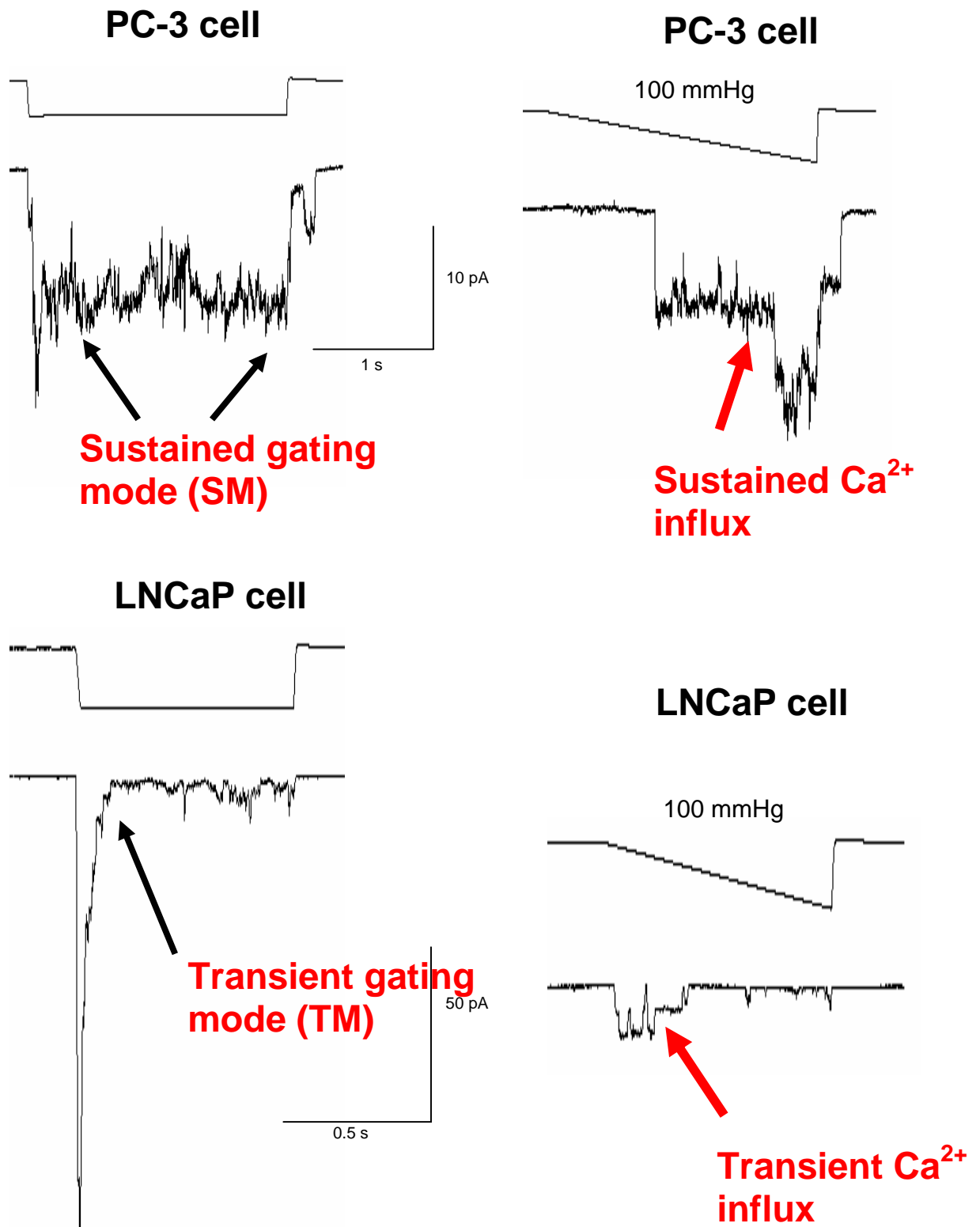


Figure 3. Different MscCa gating modes in PC-3 vs LNCaP cells. In PC3-cells, MscCa remains fully open during pressure stimuli but in LNCaP cells MscCa rapidly inactivates.

The gating of the MscCa channel is important because it determines the amount of Ca^{2+} influx in response to mechanical forces, whether they be intrinsically or extrinsically generated. Our studies indicate that there are dramatic differences between MscCa gating in PC-3 vs LNCaP cells. As indicated in Figure 3, in PC-3 cells MscCa is gated fully open for the durations of ramps of increasing membrane stretch; these might be expected to develop as the rate of forward protrusion exceeds that rate of rear retraction in the migrating cell. In comparison, in LNCaP cells, MscCa is gated in a transient mode and inactivates rapidly within 100 ms of a pressures step so that the channels may only produce a transient Ca^{2+} elevation in response to activation.

Confocal immunofluorescence studies of human prostate tumor cells.

To measure the surface distribution of TRPC1 on PC-3 cells we generated an antibody against the extracellular region of the pore domain of the TRPC1 and studied PC-3 antibody labeled cells with confocal immunofluorescence microscopy. These antibody experiments took the place of the biotinylation measurements. Figure 4 shows that TRPC1 is located at higher density on the rear tether of the cell. Because thapsigargin, a blocker of the Ca^{2+} pump located in the endoplasmic reticulum (ER), also blocks PC-3 cell migration and the development of a sustained intracellular Ca^{2+} gradient, we measured the distribution of internal Ca^{2+} stores with fluorescently-labeled thapsigargin. Figure 4 indicates that there is a higher density of ER in the cell body than in the front lamellipodium of the migrating PC-3 cell. This polarized distribution of TRPC1 and internal Ca^{2+} stores would contribute to the intracellular Ca^{2+} gradient (high rear-low front) that develops in migrating PC-3 cell (see Maroto et al. 2008).

In similar measurements made on LNCaP cells we found that both TRPC1 and internal Ca^{2+} stores are uniformly distributed at least consistent with their inability to establish a sustained intracellular Ca^{2+} gradient and undergo directional locomotion (see Maroto et al. 2008).

TASK 1.1 HAS BEEN COMPLETED AS STATED.

- 1.2 Use Westerns to establish the TRPCs (TRPC1-7) expressed in PC-3 and LNCaP cells. Use cDNA or short hairpin RNAs inserted in plasmid vectors in order to generate permanent PC-3 and LNCaP cell lines in which specific TRPCs have been either overexpressed or silenced. Use methods of 1.1 to establish the functional properties of MscCa and how TRPC1 expression and surface distribution are altered. Use time-lapse $[\text{Ca}^{2+}]_i$ imaging to study $[\text{Ca}^{2+}]_i$ dynamics and migration in the various PC cell sublines.

Time line: Year 1, months 1-12.

Milestone: Role of specific TRPCs in determining PC cell specific MscCa properties and their influence on $[\text{Ca}^{2+}]_i$ dynamics and cell migration.

Expression of TRPC1, 3-7 and TRPP2 in human prostate tumor cells

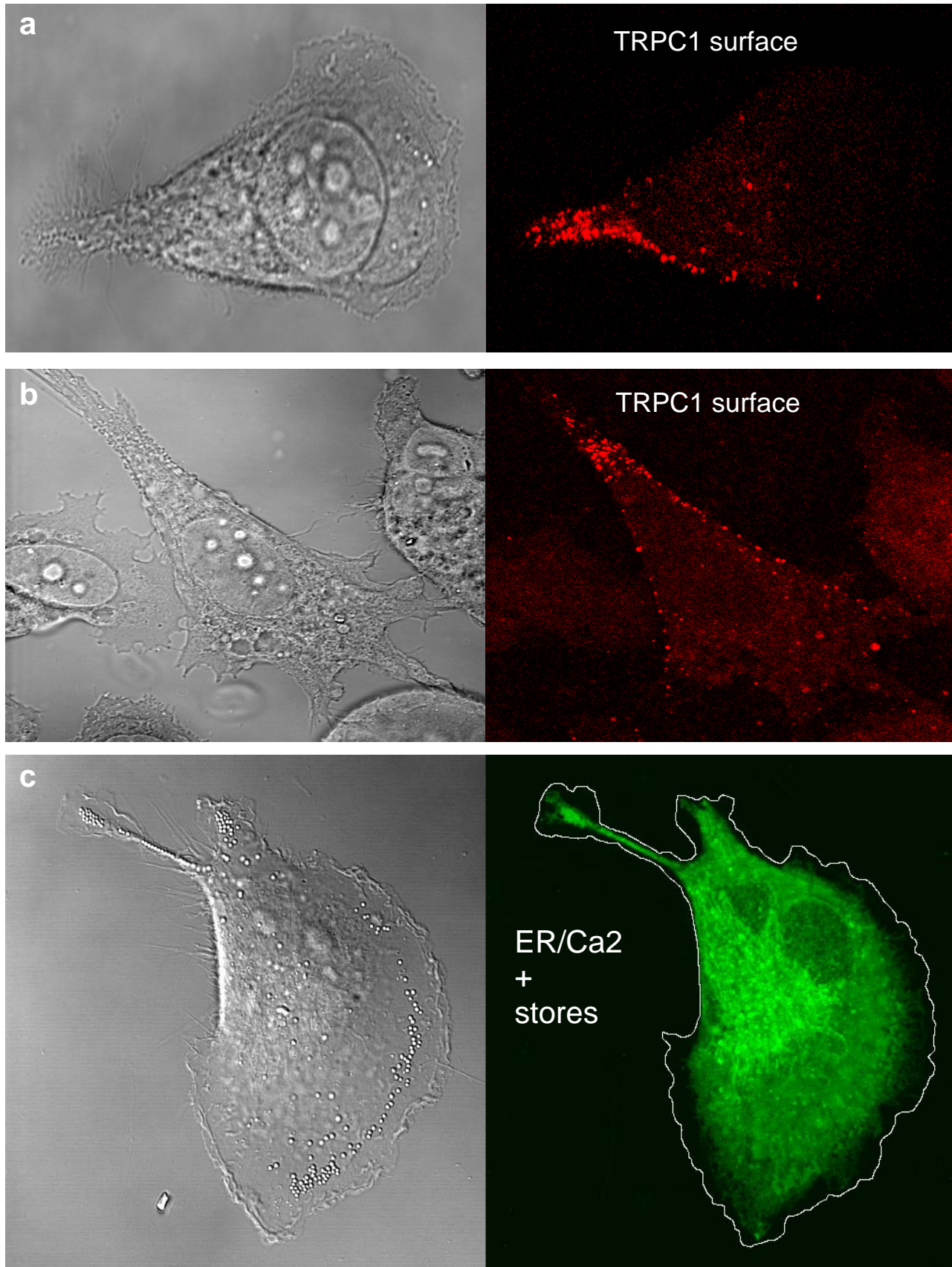


Figure 4. Photomicrographs showing the surface distribution of TRPC1 on migrating PC-3 cells (a,b) and the distribution of thapsigargin-sensitive endoplasmic reticulum internal Ca^{2+} stores (see also Maroto et al., 2008).

Table 1 summarizes the results of Western blot analysis of the different TRPCs in human prostate tumor cells. TRPC2 was not studied because the human gene contains a premature stop sequence in its open reading frame, and as a consequence is a pseudo-gene in humans. Polycystin 2, also known as TRPP2 was studied because it is expressed in human tissue and is known to interact with TRPC1. The clear cut result was that TRPC1 (and TRPC3) are expressed at higher abundance in LNCaP than in PC-3 cells, which correlates with the higher density of MscCa evident in patch clamp recordings of LNCaP vs PC-3 cells. In comparison, TRPC4 was only detected in LNCaP cells, and TRPC5 and TRPC7 were not detectable at the protein level in either PC3 or LNCaP cells using antibodies from two different commercial sources (Santa Cruz and Alamone). TRPC6 was detected in both PC-3 and LNCaP cells but was in higher abundance in PC-3 compared with LNCaP cells, which is opposite to the expression of MscCa as measured by patch clamp recording. We will also extend our current Western blot analysis of the TRPCs by measuring the relative amount of surface versus intracellular TRPCs. This is important because our recent studies indicate that over expression of TRPC1 alone does not result in its efficient trafficking to the surface membrane in CHO and COS cells when directly

Table 1: Western blot assays of TRPC1, 3-7 and TRPP2 in PC-3 and LNCaP cells

	PC3	LNCaP
TRPC1	+	++
TRPC3	+	++
TRPC4	-	+
TRPC5	-	-
TRPC6	++	+
TRPM7	-	-
Polycystin 2 (TRPP2)	+	+

compared with other channels subunits like the K^{+} channel TREK-1 (Fig. 5a,b). We also demonstrated that when TRPC1 is over expressed in the *Xenopus* oocyte (Fig. 5c), it is concentrated in the surface membrane (see Gottlieb et al., 2008 Appendix). These results indicate that exogenous TRPC1 may require specific endogenous protein subunits that combine with TRPC1 to enable it to be efficiently inserted in the cell membrane, and that this endogenous subunit(s) may be rate limiting in mammalian cells but not in the frog oocyte (Fig. 5). We are now testing the specific possibility that TRPC3 may partner with TRPC1 in prostate tumor cells to form a functional channel that is inserted in the cell membrane and regulates tumor cell migration (see below).

PC-3 cell lines in which TRPCs are suppressed or overexpressed.

So far we have shown that two PC-3 cell sublines in which TRPC1 and TRPC3 are selectively suppressed cannot migrate as measured by wound closure assay or time lapse video microscopy. Similarly, we have shown a cell line in which TRPC1 is over expressed fails to migrate. In comparison, selective suppression of TRPC4 or 6 or 7 fails to block PC3 cell migration as does over expression of TRPC3. In addition we have shown that

suppression of TRPC1 and TRPC3 prevents PC-3 cells generating an intracellular gradient and developing cell polarity.

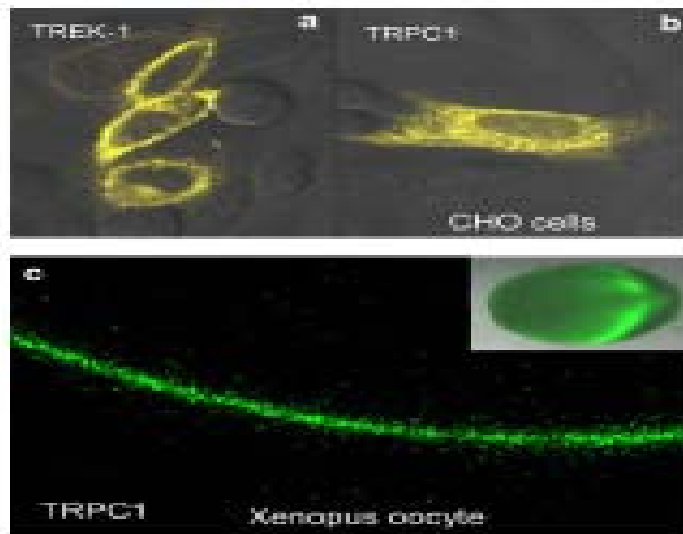


Fig.5. Confocal fluorescent microscopy of overexpressed GFP-hTRPC1 in CHO cells and frog oocytes (see Gottlieb et al. 2008 Appendix).

We are also carrying out suppression and over expression specific TRPCs in LNCaP cells but these transfections have proven more complex and time consuming, due in part to the relatively slow proliferation rates of LNCaP. So far we have our preliminary experiments indicate that suppression of TRPC3 results in LNCaP cells becoming more PC-3 like in their morphology.

TASK 1.2 IS COMPLETED WITH RESPECT TO THE PC-3 CELL SUBLINES WITH THE MAIN RESULT SHOWING THAT SUPPRESSION OF TRPC1 AND TRPC3 BLOCKS PC-3 CELL MIGRATION BUT ONLY OVER EXPRESSION OF TRPC1 (BUT NOT TRPC3) BLOCKS MIGRATION. TRPC4, 6 OR 7 DO NOT APPEAR TO PLAY A CRITICAL ROLE IN PC-3 CELL MIGRATION. OTHER ASPECTS OF THE TASK IN PARTICULAR THE TRPC SUPPRESSION AND OVEREXPRESSION IN LNCAP AND THE CHARACTERIZATION OF THE MIGRATORY BEHAVIOR WILL BE COMPLETED IN YEAR 2.

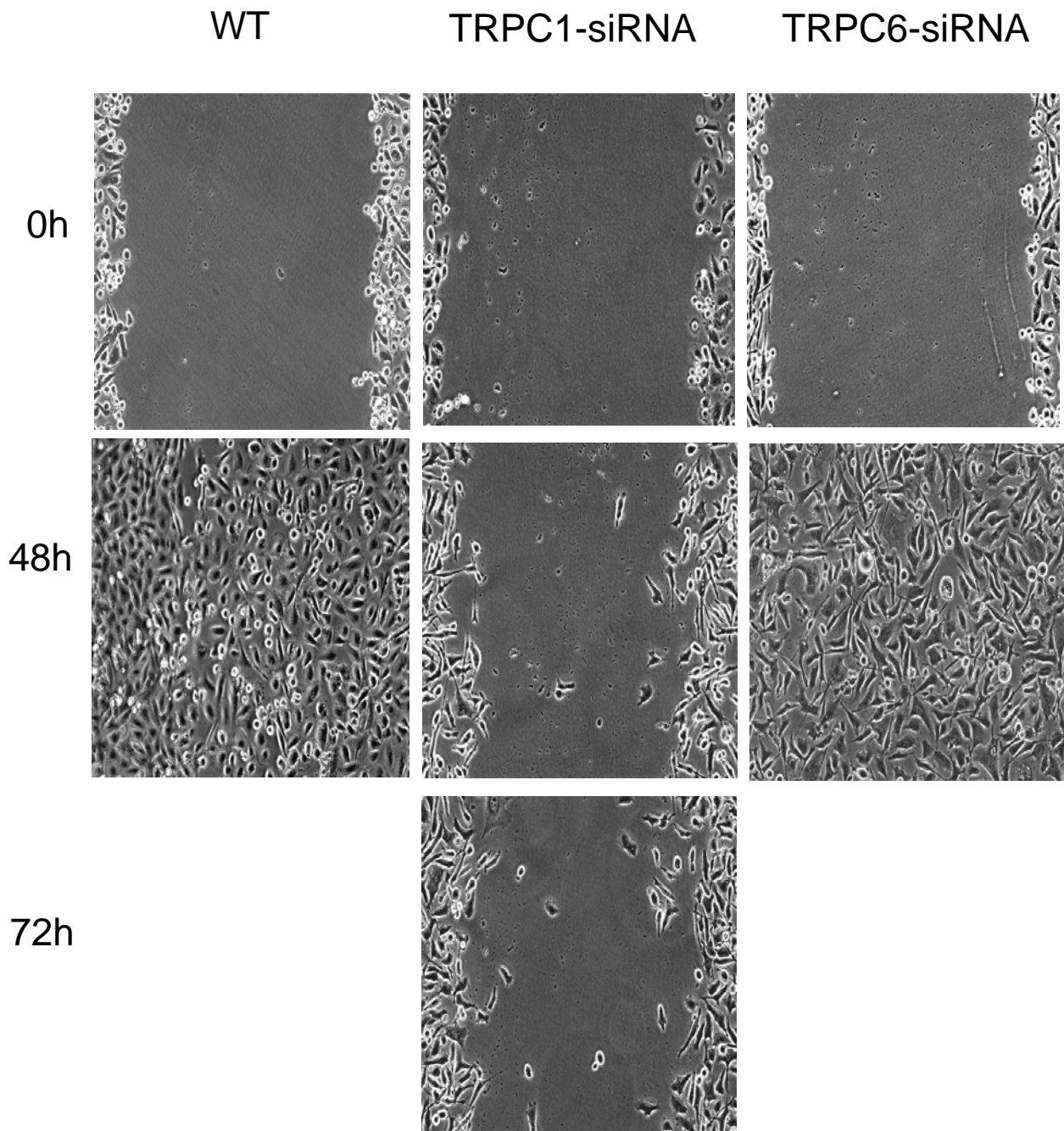


Figure 6. Wound closure assay measured for PC-3 wild type cells and PC-3 cell sublines in which TRPC1 and TRPC6 have been selectively suppressed. Although wound closure is complete within 48 hours for the wild type and TRPC6-suppressed cell line it remains incomplete after 72 hours for the TRPC1 suppressed cell line. Results obtained with TRPC3 suppression are similar to that seen with TRPC1 suppression but are not shown here.

- 1.3 Use specific agents that either promote actin depolymerization (Latrunculin A) or polymerization (jasplakinolide) to study the effects on MscCa properties and $[Ca^{2+}]_i$ dynamics and cell motility on PC cell lines.

Time line: Year 1, months 9-12; Year 2, months 1-6.

Milestone: The role of the actin-CSK in regulating MscCa properties.

The key result of our recent studies is that the transient gating of MscCa of LNCaP cells is highly sensitive to specific forms of mechanical disruption. In particular, whereas repetitive 100 ms suction pulses has no effect on the transient gating (Fig. 7), the application of 1 second pulses causes a progressive and irreversible shift of the transient gating mode to the sustained gating mode that is more PC-3 cell-like (Fig. 8).

Based on this mechanical fragility we proposed that sustained mechanical stimuli may act by disrupting the actin cytoskeleton directly underlying the membrane. However, our experiments do not support our original hypothesis, using cytochalasin D and latrunculin at concentrations (5-10 micromolar) and incubation times(> 1 hour) known to cause extensive actin depolymerization left the transient gating mode intact. Similarly, jasplakinolide (200 nM for 2 hours), an agent reported to promote actin polymerization also did not alter MscCa transient gating in LNCaP cells. However, curcumin (60 micromolar for 30 minutes) which also promotes actin polymerization reduced the transient gating mode. This result did not fit with our predictions and does not fit with lack of effect of jasplakinolide. Also against expectations we observed that 2 micromolar latrunculin altered MscCa gating in PC-3 cells so that there was an initial transient closure of channel followed by a sustained channel opening. To further complicate we have found that blebbing of the membrane of LNCaP cells using high ionic strength solution (Zhang & Hamill, 2000) does switch gating like sustained mechanical stimulation.

THE CONCLUSION FROM THESE EXPERIMENTS IS THAT AN INTACT ACTIN CYTOSKELETON IS NOT A CRITICAL REQUIREMENT FOR THE TRANSIENT GATING MODE. WE ARE CURRENTLY PURSUING AN ALTERNATIVE HYPOTHESIS THAT MECHANICAL-INDUCED CHANGES IN THE LIPID COMPOSITION OF THE BILAYER DETERMINE THE MSCCA GATING MODE (see next Task 1.4).

- 1.4 Use treatments (methyl- β -cyclodextrin with/without cholesterol) in order to deplete or enrich the bilayer with cholesterol and measure the effects on MscCa properties.

Time line: Year 2 months 6-12.

Milestone: The role of lipid bilayer structure in determining MscCa properties and PC cell motility.

As stated these experiments are to be carried out in the second year.

Deliverables: Agents that regulate MscCa properties and thereby the motility of PC cells.

Repetitive 100 ms pressure steps

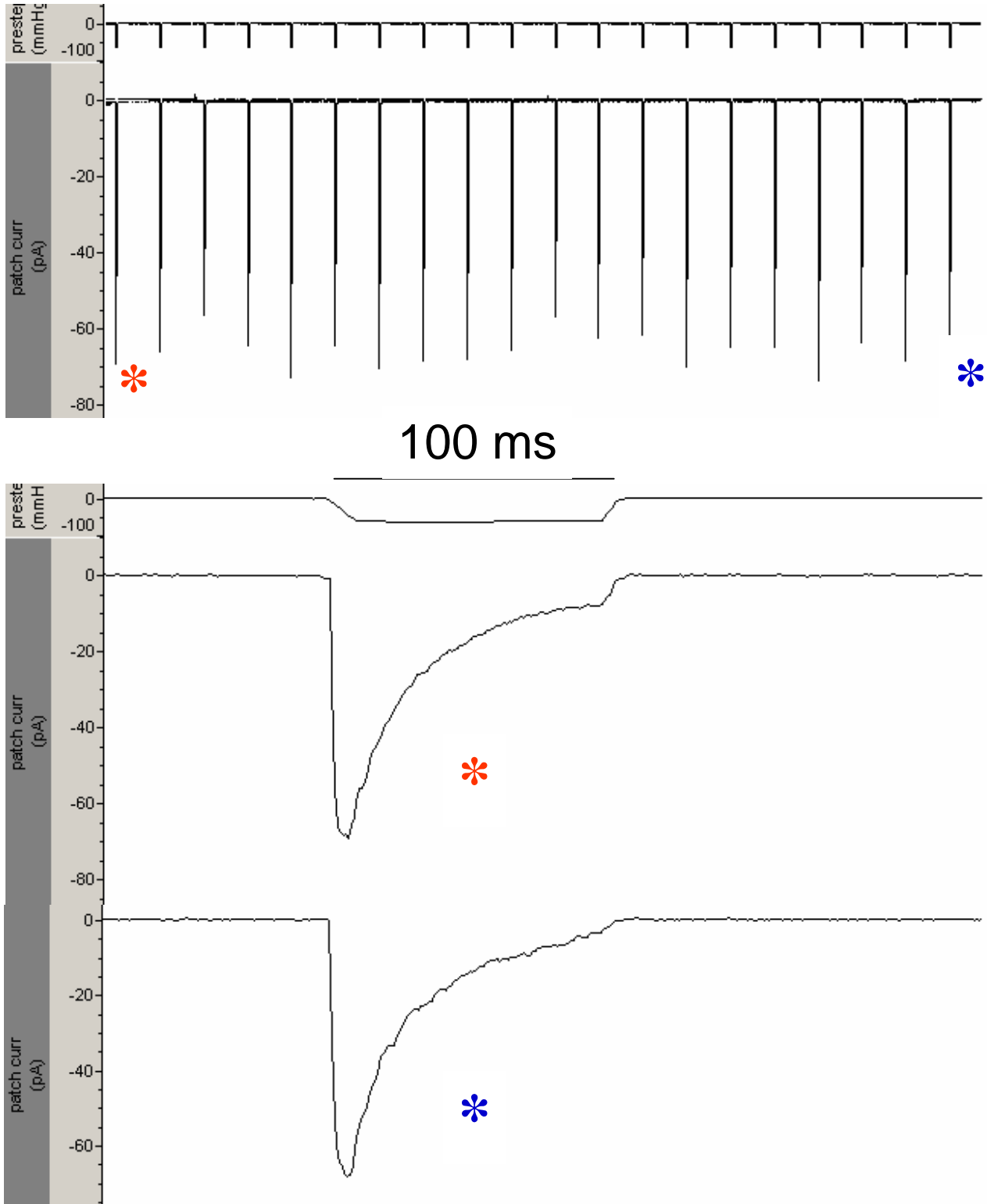


Figure 7. Patch recordings from an LNCaP cell showing that repetitive 100 ms pressure steps (top trace) does not affect the MscCa transient gating mode during the stimuli train.

Repetitive 1000 ms pressure pulses

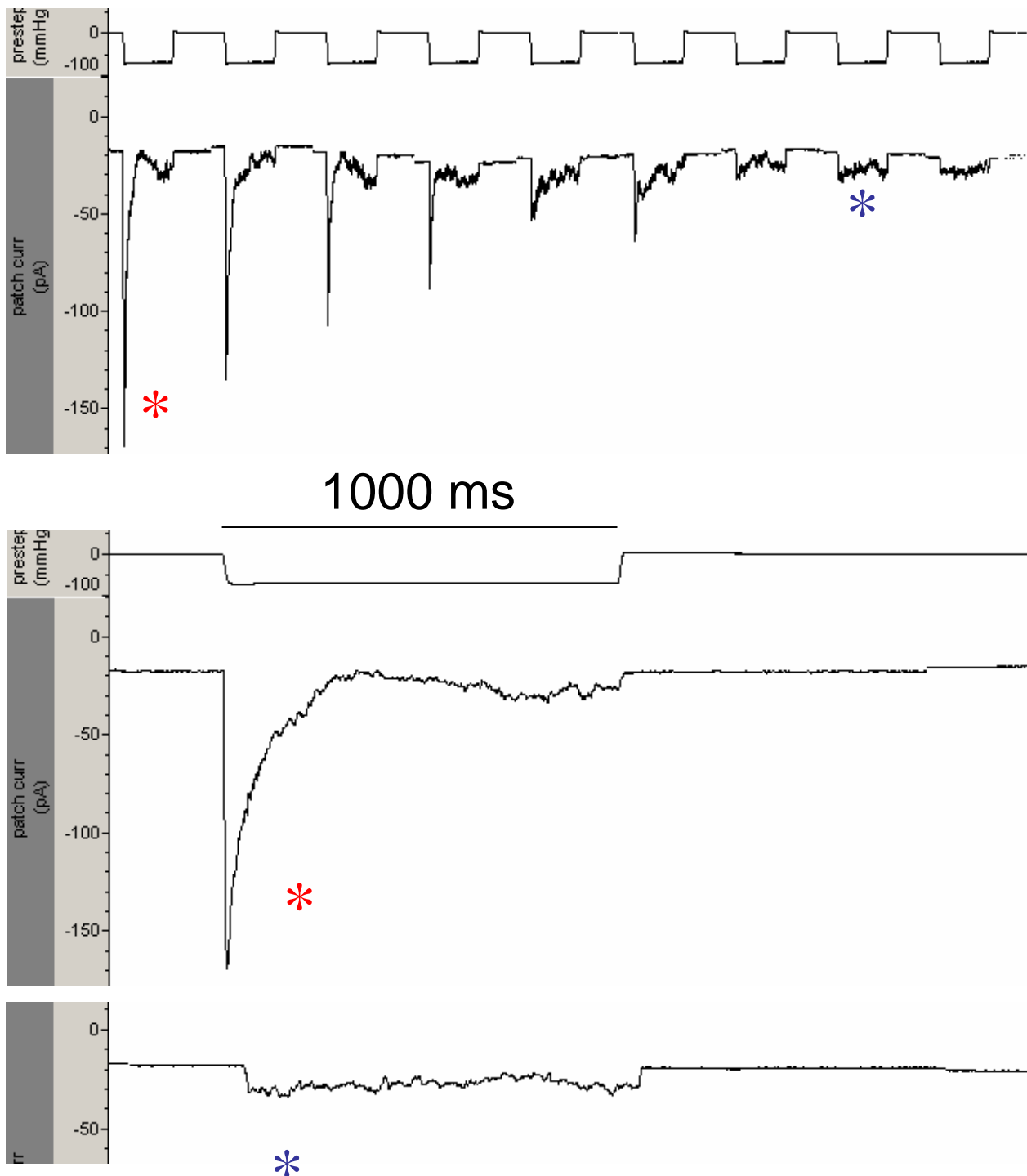


Figure 8. Patch recordings from the same LNCaP cell as in Fig. 7 indicates that repetitive 1000 ms pressures steps (top trace) causes an irreversible transition from the transient mode gating to the sustained mode. Note that even during the first response (red*) there is conversion of the gating within the latter half of the pressure pulse.

Task 2: Determine the effects of cancer-related conditions that promote tumor progression to increased invasiveness on MscCa properties.

- 2.1 Use Westerns, immunofluorescence and patch-clamp recording to examine the influence of TNF- α , a transcriptional regulator of TRPC1 expression, and TGF- β both known to promote the EMT on MscCa properties in NPE and PC cells.

Time line: Year 2, months 1-9.

Milestone: TNF- α and TGF- β regulation of MscCa/TRPC1 expression in PT cells.

As stated these experiments are to be carried out in the second year.

- 2.2 Use time-lapse Ca^{2+} imaging to determine the effects of transforming factors on the functional properties, motility and $[\text{Ca}^{2+}]_i$ dynamics of NPE and PC cells.

Time line: Year 2, months 6-12, Year 3, months 1-12.

Milestone: TNF- α and TGF- β effects on $[\text{Ca}^{2+}]_i$ dynamics and cell migration.

These experiments are to be carried out in the second year as indicated.

- 2.3 Use various conditions known to trigger increased invasiveness in the normally noninvasive LNCaP cell line to study the role of MscCa in triggering progression in these sublines.

Time line: Year 1, months 1-12; year 2 months 1-9.

Milestone: Identify cancer-linked progression factors that act on MscCa.

Deliverables: Regulator of MscCa expression in PT cells.

So far we have tested four different LNCaP cell sublines that have been reported in the literature to show increased invasiveness and/or migratory capacity over the original LNCaP parent cell line. These sublines include a subline generated by growing the LNCaP parent cell in androgen-depleted media, another subline transfected with the $\beta 3$ integrin subunit to induce $\alpha v\beta 3$ integrin expression, and two LNCaP cell sublines selected for increased invasiveness by repetitive implantation in nude mice. In each case, we have measured cell motility by time lapse video microscopy and by wound closure assay. None of the sublines were able to undergo cell migration or develop an intracellular Ca^{2+} gradient (three separate experiments in each case). Furthermore, all four sublines displayed the same transient mode of MscCa gating characteristic of the parent LNCaP cell (based on 10-50 patches in 2 to 3 experiments). The only clear cut difference was that growth in androgen depleted media resulted in significant cell death and even lower LNCaP cell growth rates. However, the cells that survived developed long neuronal processes. Our negative results seem to be consistent with the current absence of a reproducible LNCaP cell model for the transition from noninvasiveness to invasiveness. In the second year is to test whether specific growth factors (α TNF and β TGF) and substrates (e.g., fibronectin and vitronectin) can induce this transition.

Task 3: Quantify the ability of treatments that selectively target MscCa/TRPC1 and that block PC-3 cell migration in vitro to block PC-3 cell invasion when orthotopically implanted in nude mice.

- 3.1 Use time-lapse videomicroscopy and patch-clamp techniques to characterize the motility of eGFP-transfected PC-3 cells in which MScCa/TRPC1 has been selectively overexpressed or silenced.

Time line: Year 1, months 1-12.

Milestone: Genetic block of PC-3 cells migration.

We have completed this task and demonstrate that permanent suppression of TRPC1 or TRPC3 but not TRPC4, 6 or 7 blocks PC-3 cell migration (see Fig. 6 above). In addition we have shown that permanent over expression of TRPC1 but not TRPC3 blocks PC-3 cell migration.

- 3.2 Carry out orthotopic implantation of eGFP-labeled human PC-3 cells in which MscCa expression and test the effects on tumor invasion and metastasis as measured using fluorescence.

Time line: Year 2, months 6-12, year 3, months 1-12.

Milestone: Block of the PC and invasion and metastasis disease *in vivo*.

Deliverables: New gene constructs that can block PC invasion and metastasis.

Although originally planned for year 2 we have already collaborated with AntiCancer Inc. in the orthotopic implantation experiments of a TRPC1-suppressed GFP-PC-3 cell line in mice. However, in the initial step it was found that the proliferation rate of the TRPC1 suppressed PC-3 cells was so reduced compared with the normal wild types used that it was not possible to grow large enough tumors for implantation in the mouse prostate (see report form AntiCancer Incorp as Appendix 2). To overcome this problem we are testing TRPC1 suppression on more aggressive and highly proliferative PC3 cell lines.

KEY RESEARCH ACCOMPLISHMENTS.

Our key research accomplishments are as follows:

1. MscCa is expressed in human prostate tumor cells.
2. MscCa differs in its gating, density and surface distribution between invasive and noninvasive tumor cells.
3. MscCa is expressed on both the front lamellipodia and the rear tether of migrating prostate tumor cells but is found at higher density on the cell rear.
4. MscCa is expressed at a higher and uniform distribution on the non-migratory LNCaP cell line and its sublines.

5. Internal Ca^{2+} stores located in the endoplasmic reticular are more concentrated in the cell body than in the front lamellipodia of the migratory PC-3 cell but uniformly with the non invasive LNCaP cell.
6. The polarized distribution of MscCa, the sustained opening of MscCa in response to maintained stretch and the distribution of internal Ca^{2+} stores can account for the intracellular Ca^{2+} gradient (high rear-low front) that develops in migrating prostate tumor cells and determines migration directionality.
7. Suppression or over expression of TRPC1 blocks prostate tumor cell migration as measured by time-lapse video microscopy of wound closure assay.
8. Suppression of TRPC3 (but not TRPC4, 6 or 7) also blocks prostate tumor cell migration.
9. Both the magnitude and temporal characteristics of mechanical stimuli applied to the tumor cells can affect the transition between the non motile and motile MscCa gating mode.
10. Our original hypothesis that changes in the actin cytoskeleton is critical in mediating the transition in MscCa gating from the noninvasive to the invasive mode was not supported by our experiments that tested various agents known to promote either actin depolymerization or actin polymerization.
11. Different LNCaP cells sublines that have been reported to show increased invasiveness failed to show migratory behavior when measured in vitro by either wound closure assay or time lapse video microscopy. Consistent with this, all LNCaP cell sublines displayed the same inactivating MscCa gating mode characteristic of the parent cell line.
12. In addition to blocking prostate tumor cell migration, TRPC1 suppression also inhibits prostate tumor proliferation thereby blocking tumor growth when implanted subcutaneously in nude mice.

REPORTABLE OUTCOMES

The above research findings represent our reportable outcomes and major manuscript outlining these findings has been submitted for publication (Maroto et al., 2008 see Appendix 1). This manuscript is currently under revision and being prepared for resubmission based on the outcome of new in vivo experiments. The original manuscript focused entirely on our in vitro studies. However, because one reviewer requested that we carry out in vivo measurements testing the effect of TRPC1 suppression on PC-3 cell invasiveness in mice. As a result we have begun these experiments originally planned for year 2 and 3 in year 1. In addition to this manuscript we have also published four other manuscripts that describe 1) the role of different TRPCs in forming channels that are directly or indirectly activated by mechanical stimuli (Hamill & Maroto, 2007a,b), MscCa. 2) The role of MscCa in cancer progression (Maroto & Hamill, 2007). Finally, in a collaborative effort we have shown that additional protein subunits are likely needed to enable efficient trafficking of TRPC1 in mammalian cell lines (Gottlieb et al., 2008).

We also presented our results in a platform presentation at the recent IMPACT meeting in Atlanta and as a invited Speaker at 2007 Keystone meeting on Transient receptor potential channels.

CONCLUSIONS

Our ongoing experiments confirm that both MscCa and specific TRPCs (TRPC1 and TRPC3) are required for prostate tumor cell migration. In particular, their channel activity is required for the development of the intracellular Ca^{2+} gradient (high rear-low front) that determines migration directionality. Our results provide new mechanistic insight into the roles MscCa gating and surface distribution plays in maintaining the Ca^{2+} gradient in the migrating tumor cell and indicate that the MscCa gating mode and surface distribution differs between invasive and noninvasive prostate tumor cells, and that mechanical forces applied to the noninvasive cell may be able to switch gating seen in the noninvasive cell to that expressed by the invasive cell. In contradiction of our original hypothesis this mechanical switch does not depend upon changes in the underlying actin-cytoskeleton and indicates the possibility that mechanical forces act directly on the lipid bilayer and/or the channel protein itself. Experiments outlined in the original proposal will test these possibilities. Our experiments testing TRPC1 –suppression on prostate tumor cell invasion in living animals indicates that TRPC suppression may have multiple effects and reduce cell proliferation in addition to blocking cell migration.

The completion of the experiments of year 1 confirms that MscCa is a promising new target to block prostate cancer progression and provides added motivation to further understand its detailed mechanisms of action in vitro and in vivo. In particular, because Ca^{2+} influx via MscCa appears important in regulating all major modes of cell migration (i.e., mesenchymal, amoeboid and collective) it may be more effective than other strategies targeting integrins and metalloproteinases that appear to fail in vivo as a consequence of migration mode plasticity (Wolf & Friedl, 2006, Maroto & Hamill, 2007).

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LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

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CHAPTER 9

TRPCs as MS Channels

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I. OVERVIEW

This chapter reviews recent evidence indicating canonical or classical transient receptor potential (TRPC) channels are directly or indirectly mechanosensitive (MS) and can therefore be designated as mechano-operated channels (MOCs). The MS functions of TRPCs may be mechanistically related to

their better known functions as store-operated and receptor-operated channels (SOCs and ROCs). In particular, mechanical forces may be conveyed to TRPC channels through the “conformational coupling” mechanism that transmits information regarding the status of internal Ca^{2+} stores. Furthermore, all TRPCs are regulated by receptors coupled to phospholipases that are themselves MS and can regulate channels via lipidic second messengers. Accordingly, there may be several nonexclusive mechanisms by which mechanical forces may regulate TRPC channels, including direct sensitivity to bilayer mechanics, physical coupling to internal membranes, and/or cytoskeletal proteins, and sensitivity to lipidic second messengers generated by MS enzymes. Various strategies that can be used to separate out different MS-gating mechanisms and their possible role in specific TRPCs are discussed.

II. INTRODUCTION

MS ion channels transduce mechanical force into ion flux. To exhibit direct mechanosensitivity, a channel protein must be sensitive to some membrane parameter that changes with mechanical deformation. In many cases, the mechanotransduction step involves a shift in the equilibrium between closed and open channel conformations caused by changes in bilayer mechanics (e.g., lipid packing, bilayer thickness, curvature, and/or lateral pressure profile) or by direct “tugging” on the protein by cytoskeletal and/or extracellular tethers (Hamill and Martinac, 2001; Kung, 2005; Markin and Sachs, 2007; Matthews *et al.*, 2007; Powl and Lee, 2007). However, some channels may be indirectly MS in that they derive their mechanosensitivity from being functionally linked to MS enzymes that regulate the channel via second messenger or phosphorylation. Apart from mechanosensation, MS channels have been implicated in several basic cellular functions, including the regulation of cell volume, cell shape, motility, growth, and cell death. Because abnormalities in MS channels may also contribute to major human diseases, including muscular dystrophy, kidney disease, cardiac arrhythmias, hypertension, and tumor cell invasion (“mechanochannelopathies”), there is great interest in identifying the molecules that form MS channels and discovering agents that can selectively block their activity and/or expression (Chapter 4, Gottlieb *et al.*; Chapter 10, Cantiello *et al.*; Chapter 15, Drew *et al.*; Chapter 16, Lansman; Chapter 17, Maroto and Hamill; Chapter 19, Chapleau *et al.*). In eukaryotic cells, three membrane protein families, epithelial Na^+ channel (ENaC), two pore domain K^+ (TREK), and TRP families have been implicated in forming MS Na^+ (MscNa), K^+ (MscK), and cation/ Ca^{2+} (MscCa) channels, respectively (Chapter 3, Bazopoulou and Tavernarakis;

Chapter 6, Drummond; Chapter 7, Chemin *et al.*; Chapter 8, Castiglioni and García-Añoveros; Chapter 10, Cantiello *et al.*). Here, we focus on the TRPCs, which have been implicated in forming the ubiquitous stretch-activated MscCa (Maroto *et al.*, 2005).

III. PRACTICAL ASPECTS OF RECORDING MS CHANNELS

The most direct method to determine if an ion channel is MS is to apply a hydrostatic or osmotic pressure gradient across the membrane patch while monitoring single-channel currents (Hamill *et al.*, 1981; Hamill, 1983; Guharay and Sachs, 1984; Hamill, 2006). This method led directly to the discovery of MscK and MscCa in frog red blood cells and cultured chick myotubes, respectively (Hamill, 1983; Guharay and Sachs, 1984). Subsequently, MscK and MscCa were shown to be widely expressed in sensory and nonsensory animal cells and proposed to function in various physiological processes including regulatory volume decrease (RVD) in response to osmotic swelling (Sachs, 1988; Morris, 1990; Sackin, 1995; Sachs and Morris, 1998; Hamill and Martinac, 2001; Patel and Honore, 2001). In several cases of RVD, it was possible to demonstrate that the same channel (e.g., MscK, MscCa, MscL, and MscS) was activated by cell swelling and membrane stretch (Christensen, 1987; Sackin, 1989; Cemerikic and Sackin, 1993; Levina *et al.*, 1999; Vanoye and Reuss, 1999). However, in other cases, most notably the vanilloid transient receptor potential 4 (TRPV4), the channel was sensitive to cell volume changes without displaying stretch sensitivity (Strotmann *et al.*, 2000). This discrepancy may arise because TRPV4 is not directly MS but instead derives its volume sensitivity from being coupled to one or more MS enzymes (Watanabe *et al.*, 2003; Xu *et al.*, 2003; Vriens *et al.*, 2004; Cohen, 2005a). In particular, one group has proposed that TRPV4 is coupled to an osmotic-sensitive Src protein tyrosine kinase that regulates channel activation by tyrosine phosphorylation (Xu *et al.*, 2003; Cohen, 2005b). Another group (Watanabe *et al.*, 2003; Vriens *et al.*, 2004) has proposed that TRPV4 is coupled to the volume-sensitive phospholipase A₂ (PLA₂; Basavappa *et al.*, 1988; Lehtonen and Kinnunen, 1995) that releases arachidonic acid (AA) from membrane phospholipids, which is then metabolized, via the action of cytochrome P450, into 5',6'-epoxyeicosatrienoic acid (5',6'-EET). In support of the latter scheme, it was shown that blocking either PLA₂ or cytochrome P450 inhibits TRPV4 activation, whereas direct application of 5',6'-EET activates TRPV4 in a membrane-delimited manner (Watanabe *et al.*, 2003; Vriens *et al.*, 2004). The group that carried out the PLA₂ study was unable to reproduce the Src results (Cohen, 2005b), indicating the mechanism(s) that activates TRPV4 may vary with cell type and/or experimental conditions.

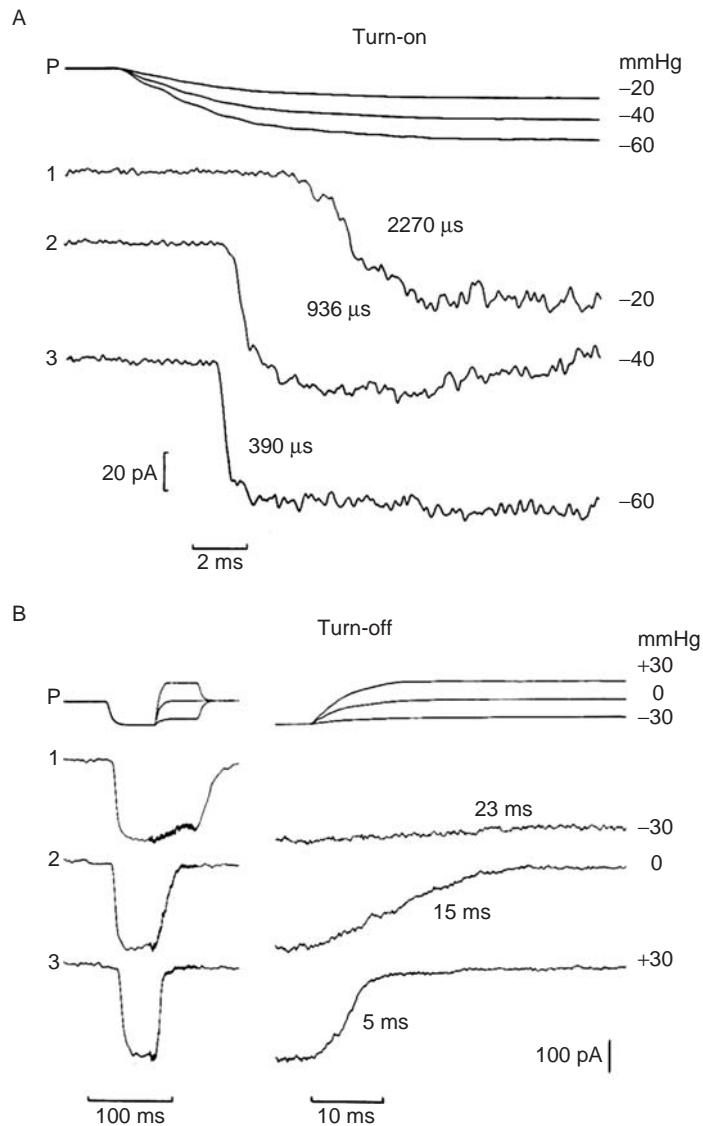


FIGURE 1 Fast turn-on and turn-off of MS channel currents measured in response to suction steps applied with a pressure clamp. A shows in the top trace (labeled P) three superimposed suction steps of -20, -40, and -60 mmHg applied to a cell-attached patch on a *Xenopus* oocyte. The lower three traces show the change in latency and the rate of turn-on of the currents in response to the increasing suction steps. The numbers in microseconds alongside each trace reflect the time from 20% to 80% of the peak current. B shows recordings designed to show the pressure dependence of the current turn-off. In the left-hand panel, the

In any case, the results indicate that while TRPV4 may function as a mechano-effector, it is not directly MS (O'Neil and Heller, 2005). There are added complications with other channels because they can be activated both by membrane stretch and by lipidic second messengers including AA and lysophospholipids (Martinac *et al.*, 1990; Kim, 1992; Hamill and McBride, 1996; Casado and Ascher, 1998; Patel *et al.*, 2001; Chapter 7, Chemin *et al.*). In this case, the issue becomes how to distinguish between direct and indirect mechanisms of mechanosensitivity.

IV. DISTINGUISHING DIRECT VS INDIRECT MS CHANNELS

Channels that are directly MS should only be limited by the conformational transitions of the channel protein, and may therefore be activated and deactivated with relatively brief delays (i.e., in the submillisecond or millisecond range). In comparison, channels dependent on enzymatic reactions and/or diffusion of second messenger may be expected to show much longer delays in opening and closing (e.g., ≥ 1 s). Figure 1 illustrates the activation and deactivation of the oocyte MS channel in response to increasing pressure steps. The transition time for the pressure step is limited by the speed of the pressure clamp (McBride and Hamill, 1992; 1995, 1999; Besch *et al.*, 2002). However, once the threshold pressure for activation is reached, the MS current turns on in few hundred microseconds (Fig. 1A). With increasing step size, both the latency and rise time of the MS current decreases consistent with the pressure reaching threshold faster. Similarly, the current turn-off indicates the channels close faster with larger pressure steps (Fig. 1B). The slower time for turn-off compared with turn-on presumably reflects the relatively slower rate of MS channel closure under these conditions (Fig. 1B). Similar brief delays and fast channel opening have been reported for activation and deactivation of the expressed a TWIK (tandem of P domains in a weak inward rectifier K^+ channel)-related arachidonic acid stimulated K^+ channel (TRAAK) (Honoré *et al.*, 2006). In contrast, an MscK expressed in snail neurons, which like TRAAK is a two-pore domain K^+ channel (Vandorpe and Morris, 1992), shows activation delays of up to several seconds (Small and Morris, 1994). However, because the delays can

superimposed suction pulse waveforms are shown in the upper trace and the corresponding current responses are shown in the lower traces. The initial activating suction was -40 mmHg for all three pulses. To turn off the currents the suction was stepped back to three increasing positive pressures. The right-hand panel shows on an expanded timescale the regions of the turn-off that were highlighted in the left panel with the numbers in microseconds representing the turn-off times (20–80%), and indicate that as the turn-off step size increases the channels turn off faster (Reproduced from McBride and Hamill, 1993).

be abolished by mechanical or chemical disruption of the cytoskeleton (CSK), they presumably arise from CSK constraint of the bilayer that prevents rapid transmission of tension to the channel. So far, studies measuring possible delays in pressure activation of TRPs that are suspected of being indirectly MS have not been performed. In the case of TRPV4, which has been functionally linked to PLA₂ (Vriens *et al.*, 2004), it will be interesting to determine whether its apparent lack of stretch sensitivity when measured in the patch was overlooked because of long delays and slow channel activation in response to applied pressure.

A further strategy for discriminating between direct and indirect MS channel mechanisms is to use specific inhibitors to test for involvement of MS enzymes (e.g., *p*-bromophenacyl bromide for PLA₂, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine (PP2) for Src tyrosine kinase, and U73122 for phospholipase C (PLC). In particular, the stretch sensitivity of the MS channel in arterial smooth muscle has been reported to be abolished by the PLC inhibitor U73122 (Park *et al.*, 2003). Furthermore, Ca²⁺ influx in dystrophic muscle that is mediated by a TRPC-dependent SOC and/or MOC (Vandebrouck *et al.*, 2002; Ducret *et al.*, 2006) can be abolished by inhibitors of PLA₂ (Lindahl *et al.*, 1995; Boittin *et al.*, 2006; Section VIII.A.3).

The most unequivocal method for distinguishing direct from indirect mechanosensitivity is to examine whether the detergent-solubilized channel protein retains stretch sensitivity when reconstituted in pure liposomes. So far, this test has been applied to several MS channels in prokaryotes and MscCa expressed in the frog oocyte (Sukharev *et al.*, 1993; Kloda and Martinac, 2001a,b; Sukharev, 2002; Maroto *et al.*, 2005). This approach also offers the potential of definitive evidence on whether lipidic second messengers [e.g., diacylglycerol (DAG), AA, lysophospholipids and 5',6'-EET] activate the channel by binding directly to the channel protein and/or its surrounding lipid without intermediate steps. Furthermore, the same method may also be applied to determine whether multiprotein component MS-signaling complexes can be functionally reconstituted from their specific elements (e.g., TRPV4, PLA₂, and so on).

Although stretch sensitivity measured in the patch can be used to demonstrate a channel protein is MS at the biophysical level, it cannot prove the channel functions as a physiological mechanotransducer (Hamill, 2006). Indeed, many structurally diverse voltage- and receptor-gated channels [e.g., Shaker, L-type Ca²⁺ channels, *N*-methyl-D-aspartate receptor (NMDAR), S-type K⁺ channels], as well as the simple model peptide channels alamethicin and gramicidin A, display stretch sensitivity in patch recordings (Opsahl and Webb, 1994; Paoletti and Ascher, 1994; Martinac and Hamill, 2002; Chapter 11, Morris and Juranka). In order to demonstrate functionality, one also needs to show that blocking the channel (pharmacologically and/or genetically) inhibits a mechanically induced cellular/physiological process.

V. EXTRINSIC REGULATION OF STRETCH SENSITIVITY

Stretch sensitivity is unlikely to be accounted for by a single structural domain analogous to the S-4 voltage sensor-domain shared by voltage-gated Na^+ , K^+ , and Ca^{2+} channels (Hille, 2001); even the relatively simple peptide channels, gramicidin and alamethicin, which have dramatically different structures and gating mechanisms, exhibit stretch sensitivity (Hamill and Martinac, 2001; Martinac and Hamill, 2002). Furthermore, stretch sensitivity is not a fixed channel property but rather can undergo significant changes with changing extrinsic conditions. For example, mechanical and/or chemical disruption of the CSK can either enhance or abolish the stretch sensitivity of specific channels (Guharay and Sachs, 1984; Hamill and McBride, 1992, 1997; Small and Morris, 1994; Patel and Honore, 2001; Hamill, 2006); changes in bilayer thickness (Martinac and Hamill, 2002), membrane voltage (Gu *et al.*, 2001; Chapter 11, Morris and Juranka), or dystrophin expression (Franco-Obregon and Lansman, 2002; Chapter 16, Lansman) can switch specific MS channels from being stretch-activated to stretch-inactivated; specific lipids (Patel and Honore, 2001; Chemin *et al.*, 2005), nucleotides (Barsanti *et al.*, 2006a and references therein), and increased internal acidosis (Honore *et al.*, 2002; Barsanti *et al.*, 2006b) can convert MS channels into constitutively open “leak” channels. The basis for these changes is often because changes in the bilayer, CSK, and/or ECM alter how mechanical forces are conveyed to the channel protein. The practical consequence may be that the specific conditions associated with reconstitution and/or heterologous expression may alter the stretch sensitivity of the reconstituted/expressed channel.

VI. STRATEGIES TO IDENTIFY MS CHANNEL PROTEINS

Once a channel has been functionally identified as stretch sensitive, there are several strategies that can be used to identify the membrane protein. The first strategy of “expression cloning” involves generating a cDNA library from cells expressing the channel, and then screening the library, typically in *Xenopus* oocytes or a mammalian cell line. This strategy has been used to clone several voltage- and receptor-gated channels, including the first vanilloid receptor TRP channel TRPV1 (Caterina *et al.*, 1997). However, its application to MS channels has proven problematic because the expression vehicles express their own endogenous MS channels. The second strategy of “functional protein reconstitution” involves detergent solubilizing and reconstituting membrane proteins into liposomes and then screening for stretch sensitivity using patch clamp recording. This strategy has been used to successfully identify/clone a number of MS channel proteins from bacteria and archaea

(Sukharev *et al.*, 1993, 1994; Sukharev, 2002; Martinac, 2007). It was also used to implicate a TRPC in forming MscCa (Maroto *et al.*, 2005).

VII. GENERAL PROPERTIES OF TRPCs

This section provides an overview of the TRPC subfamily (for reviews see Minke and Cook, 2002; Vazquez *et al.*, 2004a; Montell, 2005; Nilius and Voets, 2005; Parekh and Putney, 2005; Owsianik *et al.*, 2006). The first TRP was discovered in a *Drosophila* mutant that showed a transient rather than a sustained receptor potential in response to light (Cosens and Manning, 1969; Minke *et al.*, 1975; Montell and Rubin, 1989). On the basis of these kinetics, the protein was designated TRP. Subsequently seven mammalian TRP homologues were discovered that together with TRP now make up the TRPC1–7. Other TRP subfamilies include TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPM (melastatin), TRPML (mucolipid), TRPN (NompC), and TRPY (yeast), and these together with TRPCs form the TRP superfamily. In addition to MS TRPCs, specific members of the other subfamilies have also been implicated in mechanotransduction so that the MS mechanisms discussed below may be general (Walker *et al.*, 2000; Palmer *et al.*, 2001; Zhou *et al.*, 2003; Nauli and Zhou, 2004; O’Neil and Heller, 2005; Voets *et al.*, 2005; Saimi *et al.*, 2007). The proposed transmembrane topology of TRPCs is reminiscent of voltage-gated channels—sharing six transmembrane-spanning helices (TM1–6), cytoplasmic N- and C-termini, and a pore region between TM5 and TM6—but lacking the positively charged residues in the TM4 domain that forms the voltage sensor. The seven mammalian TRPC channels also share an invariant sequence in the C-terminal tail called a TRP box (E-W-K-F-A-R), as well as 3–4 N-terminal ankyrin repeats. Although the ankyrin repeats may act as gating springs for MS channels (Howard and Bechstet, 2004; Saimi *et al.*, 2007; see also Chapter 8, Castiglioni and García-Añoveros; Chapter 10, Cantiello *et al.*), their exact role and that of the TRP box remains to be verified (Vazquez *et al.*, 2004a; Owsianik *et al.*, 2006). The TRPCs share very little sequence identity in the region that is C-terminal of the TRP box, except for the common feature of CaM- and IP₃R-binding domains that have been implicated in Ca²⁺ feedback inhibition and activation by store depletion, respectively (Kiselyov *et al.*, 1998; Vaca and Sampieri, 2002). On the basis of sequence homology, the TRPCs have been divided into two major subgroups TRPC3/6/7 (70–80% homology) and TRPC1/4/5 (65% homology). TRPC2 is in a special class because multiple stop codons within its open reading frame make it a pseudogene in humans. However, it does form a functional channel in rodents (Section VIII.B).

A. TRPC Expression

TRPCs are widely expressed in mammalian tissues, with some human cells expressing all six and others expressing only one or two (Riccio *et al.*, 2002; Antoniotti *et al.*, 2006; Goel *et al.*, 2006; Hill *et al.*, 2006). The latter cells may prove useful for dissecting out specific TRPC functions, but it is necessary that selective expression be verified at both transcriptional and protein levels, since low turnover proteins may require little mRNA, and high mRNA levels need not translate into high membrane protein levels (Andersen and Seilhamer, 1997). Another caveat is that TRPC expression patterns may vary during development and with culture conditions (e.g., presence or absence of growth factors). For example, TRPC1 expression is upregulated by (1) serum deprivation where it contributes to increased proliferation of pulmonary arterial smooth muscle cells (Golovina *et al.*, 2001), (2) tumor necrosis factor α where it enhances endothelial cell death (Paria *et al.*, 2003), and (3) vascular injury *in vivo* which contributes to human neointimal hyperplasia (Kumar *et al.*, 2006); TRPC6 expression in pulmonary arterial smooth muscle cells is enhanced by platelet-derived growth factor and by idiopathic pulmonary arterial hypertension (Yu *et al.*, 2003, 2004).

B. TRPC Activation and Function

Studies of TRPC activation and function are complicated by their polymodal activation and splice variants that display different activation mechanisms (Ramsey *et al.*, 2006). However, all TRPCs are regulated by PLC-coupled receptors (i.e., G-protein-coupled receptors or tyrosine kinase receptors). PLC hydrolyzes a component of the bilayer, PIP₂, into two distinct messengers—the soluble InsP₃ that activates the IP₃R in the ER to release Ca²⁺ from internal stores and the lipophilic DAG that may regulate TRPs indirectly via protein kinase C (PKC) or by interacting directly with the TRPCs in a membrane-delimited manner (Delmas *et al.*, 2002; Clapham, 2003; Ramsey *et al.*, 2006). Furthermore, Bolotina and colleagues have shown that a diffusible second messenger produced by depletion of Ca²⁺ stores activates a Ca²⁺ independent phospholipase (iPLA₂) that generates lysophospholipids, which are themselves capable of activating SOCs when exogenously applied to inside-out patches (Smani *et al.*, 2003; Bolotina and Csutora, 2005). Therefore, although all TRPCs could be classified as ROCs (but see Janssen and Kwan, 2007), they are more often subdivided into either SOCs, based on their sensitivity to Ca²⁺ store depletion that may or may not depend on PLC–IP₃R signaling, or ROCs

that are activated by DAG or its byproducts, but are insensitive to Ca^{2+} store depletion (Hofmann *et al.*, 1999). To be classified as a SOC, the channel should be gated by a variety of procedures that only share the common feature of reducing Ca^{2+} stores (Parekh and Putney, 2005). Unfortunately, there have been conflicting reports for all seven TRPCs on whether they function as SOCs, ROCs, or both. Here, we focus on a further complication that the same mechanisms that make a channel a SOC or a ROC may also contribute to it being MS.

C. TRPC-TRPC Interactions

If all 7 TRPC subunits are expressed in a given cell and 4 subunits are required to form a channel (i.e., homotetrameric and heterotetrameric), then there could be as many as 100 different TRPC channels types. However, the number would be smaller if only certain TRPC-TRPC combination can occur. Two different models for TRPC interactions have been proposed: a homotypic model in which only subunits within each subfamily can interact to form channels with TRPC1/4/5 forming SOCs and TRPC3/6/7 forming ROCs (Hofmann *et al.*, 2002; Sinkins *et al.*, 2004), and a heterotypic model that also allows interactions between subfamily members, in this case with TRPC1, TRPC3, and TRPC7 proposed to form SOCs (i.e., without TRPC4 and TRPC6) and TRPC3, TRPC4, TRPC6, and TRPC7 proposed to form ROCs (without TRPC1; Zagranichnaya *et al.*, 2005). In the heterotypic model, TRPC1's role is limited to SOCs and TRPC4's and TRPC6's roles are limited to ROCs, while TRPC3 and TRPC7 can participate as both SOCs and ROCs (Zagranichnaya *et al.*, 2005). Interestingly, both models were generated from studies of the human embryonic kidney cell line, HEK-293, with the homotypic model based on gain-of-function (i.e., from TRPC overexpression) and the heterotypic model based on loss-of-function (i.e., from TRPC suppression). However, one complication with the former approach is that the level of TRPC expression can determine channel function. In particular, it has been shown that low TRPC3 expression result in SOCs, while high expression result in ROCs (Vazquez *et al.*, 2003). This effect presumably occurs because high expression promotes homomeric TRPC3 channels, whereas low levels allow for heteromers that include endogenous subunits (Brereton *et al.*, 2001; Vazquez *et al.*, 2003). Differences in channel function may also arise depending on whether the cell is permanently or transiently transfected, presumably because stable transfection allows time adaptive changes in endogenous protein expression (Lievremont *et al.*, 2004).

D. TRPC Interactions with Scaffolding Proteins

TRPCs also interact with a variety of regulatory and scaffolding proteins that may add further diversity and segregation of the channels (Ambudkar, 2006). In particular, it has been shown that several TRPCs assemble into multiprotein and lipid-signaling complexes that result in physical and functional interactions between the plasma membrane and CSK and ER resident proteins. These interactions may also allow for mechanical forces to be conveyed via a tethered mechanism to gate the channel (Howard *et al.*, 1988; Hamill and Martinac, 2001; Matthews *et al.*, 2007; Chapter 3, Bazopoulou and Tavernarakis; Chapter 10, Cantiello *et al.*). Alternatively, the interactions may also serve to constrain the development or transmission of bilayer tension to the TRPC and thereby “protect” it from being mechanically activated (Small and Morris, 1994; Hamill and McBride, 1997). For all TRPCs, the C-terminal coiled-coil domains and N-terminal ankyrin repeats have the potential to mediate protein–CSK interactions. All TRP family members also encode a conserved proline-rich sequence LP(P/X)PFN in their C-termini that is similar to the consensus-binding site for Homer, a scaffold protein that has been shown to facilitate TRPC1 interaction with IP₃R—disruption of which has been proposed to promote SOC activity (Yuan *et al.*, 2003). For example, TRPC1 mutants lacking Homer protein-binding sites show diminished interaction between TRPC1 and IP₃R and the TRPC1 channels are constitutively active. Similarly, coexpression of a dominant-negative form of Homer increases basal TRPC1 channel activity (Yuan *et al.*, 2003). Another protein I-mfa, which inhibits helix-loop-helix transcription factors, also binds to TRPC1 and blocks SOC function (Ma *et al.*, 2003). TRPC1 also expresses a dystrophin domain in its C-terminus (Wes *et al.*, 1995) that may allow interaction with dystrophin, and possibly explain why the absence of dystrophin in Duchenne muscular dystrophic muscle results in TRPC1 channels being abnormally gated open (Section VIII.A.3). TRPC1 also shows a putative caveolin-1-binding domain that may promote its functional recruitment into lipid rafts and increase SOC activity (Lockwich *et al.*, 2000; Brazier *et al.*, 2003; Ambudkar, 2006). TRPC1 has been shown to interact with stromal interaction molecule (STIM), the putative ER Ca²⁺ sensor that can apparently regulate TRPC1 SOC function (Huang *et al.*, 2006). Juncate is another IP₃R-associated protein, and it interacts with TRPC2, TRPC3, and TRPC5 (but not TRPC1) to regulate their SOC/ROC function (Treves *et al.*, 2004; Stambouliau *et al.*, 2005). In pulmonary endothelial cells, TRPC4 is localized to cell–cell adhesions in cholesterol-rich caveolae and has been shown to interact with the spectrin CSK via the protein 4.1 (Torihashi *et al.*, 2002; Cioffi *et al.*, 2005).

Furthermore, either deletion of the putative 4.1 protein-binding site on the TRPC4 C-terminus of TRPC4 or addition of peptides that competitively bind to that site are able to reduce SOC activity. Another site for TRPC4-CSK interaction involves the PSD-95/disk-large protein/zona occludens 1 (PDZ)-binding domain located at the TRPC4 distal C-terminus that binds to the Na^+/H^+ exchange regulatory factor (NHERF)-scaffolding protein (Tang *et al.*, 2000; Mery *et al.*, 2002). TRPC6 interacts with the stomatin-like protein podocin that may modulate its MOC function in the renal slit diaphragm (Reiser *et al.*, 2005). Interestingly, another stomatin homologue, MEC-2, links the putative MS channel to the microtubular CSK in *Caenorhabditis elegans* neurons (Chapter 3, Bazopoulou and Tavernarakis).

In summary, TRPCs undergo dynamic interactions with various scaffolding proteins that may act to inhibit or promote a particular mode of channel activation. Any one of these interactions may be important in modulating the mechanosensitivity of TRPC by focusing mechanical force on the channel or constraining the channel and/or bilayer from responding to mechanical stretch. It may be that the right combination of TRPC proteins and accessory proteins are needed to produce channels that are not constitutively active but are responsive to store depletion and/or mechanical stimulation.

E. Single TRPC Channel Conductance

Single-channel conductance provides a good identifying fingerprint of specific channels. However, compared with whole-cell current recording studies, there have been relatively few studies of the single-channel currents that are either enhanced or deleted by TRPC overexpression or suppression, respectively. Furthermore, there is no simple way to determine if a channel reflects a homomeric rather than a heteromeric TRPC. Another practical issue for comparisons has been the lack of standardized recording conditions. Nevertheless, a survey of the TRPC single-channel literature indicates the following order for conductance values $\text{TRPC3 (65 pS)} > \text{TRPC5 (50 pS)} > \text{TRPC4 (32 pS)} \sim \text{TRPC6 (31 pS)} > \text{TRPC1 (\sim 20 pS)}$ for estimates made from cell-attached recordings with 100- to 150-mM Na^+/Cs^+ , 1- to 4-mM $\text{Ca}^{2+}/\text{Mg}^{2+}$ at -40 to -100 mV (Hurst *et al.*, 1998; Kiselyov *et al.*, 1998; Hofmann *et al.*, 1999; Yamada *et al.*, 2000; Liu *et al.*, 2003; Strübing *et al.*, 2003; Bugaj *et al.*, 2005; Maroto *et al.*, 2005; Inoue *et al.*, 2006). The only available estimates for TRPC2 (42 pS) and TRPC7 (60 pS) were made with no divalents (Perraud *et al.*, 2001; Zufall *et al.*, 2005). These numbers may serve as a baseline for the future conductance measurements of the purified/reconstituted TRPCs.

F. TRPC Pharmacology

The pharmacological tools to study TRPCs are limited with the following agents reported to block, stimulate, or have no effect on different TRPCs (Ramsey *et al.*, 2006): SKF-96365 blocks TRPC3 and TRPC6 currents (at $\sim 5 \mu\text{M}$), and is considered a ROC more than a SOC blocker; 2APB (2-aminoethoxydiphenyl borate) blocks TRPC1 (80 μM), TRPC5 (20 μM), and TRPC6 (10 μM) but not TRPC3 (75 μM), and is considered more a SOC than a ROC blocker; Gd^{3+} and La^{3+} block TRPC1 and TRPC6, but potentiate TRPC4 and TRPC5 (in micromolars; Jung *et al.*, 2003); flufenamate blocks TRPC3, TRPC5, and TRPC7 (100 μM), but potentiates TRPC6; and tarantula venom peptide, GsmTX4, (Gottlieb *et al.*, 2004) blocks TRPC1 in mammalian cells but not in *Xenopus* oocytes (Hamill, 2006; Chapter 4, Gottlieb *et al.*). Other agents of interest that need to be systematically tested on both SOC and ROC activity include gentamicin, ruthenium red, GsmTX4, and amiloride (Lane *et al.*, 1991, 1992; R  sch *et al.*, 1994; Flemming *et al.*, 2003; Suchyna *et al.*, 1998, 2004; Jacques-Fricke *et al.*, 2006).

VIII. EVIDENCE FOR TRPC MECHANOSENSITIVITY

Below, we consider the MS role of specific TRPCs. At this time, the main evidence exists for TRPC1 (SOC), TRPC6 (a DAG-activated ROC), and to a lesser extent TRPC4 (an AA-activated ROC). However, as discussed in Section IX, a basic issue is whether the mechanisms that confer SOC and ROC activity on TRPC channels also contributes to there mechanosensitivity. In this case, all TRPs may end up expressing some degree of mechanosensitivity.

A. TRPC1

TRPC1 was the first identified vertebrate TRP homologue (Wes *et al.*, 1995; Zhu *et al.*, 1995) and initial heterologous expression of human TRPC1 (hTRPC1) in Chinese hamster ovary (CHO) and sf9 cells enhanced SOC currents (Zitt *et al.*, 1996). However, a subsequent study indicated hTRPC1 expression in sf9 cells induced a constitutively active nonselective cation channel that was not sensitive to store depletion (Sinkins *et al.*, 1998). This early discrepancy raises the possibility that store sensitivity (and perhaps stretch sensitivity) may depend on a variety of conditions (e.g., expression levels, presence of endogenous TRPCs, and state of phosphorylation). For example,

TRPC1 has multiple serine/threonine phosphorylation sites in the putative pore-forming region and the N- and C-termini, and at least one report indicates that PKC α -dependent phosphorylation of TRPC1 can enhance Ca²⁺ entry induced by store depletion (Ahmmed *et al.*, 2004). Despite this early discrepancy, many studies now point to TRPC1 forming a SOC (Liu *et al.*, 2000, 2003; Xu and Beech, 2001; Kunichika *et al.*, 2004; for reviews see Beech *et al.*, 2003; Beech, 2005) and in cases where TRPC1 expression has not resulted in enhanced SOC (Sinkins *et al.*, 1998; Lintschinger *et al.*, 2000; Strübing *et al.*, 2001), it has been argued that TRPC1 was not trafficked to the membrane (Hofmann *et al.*, 2002). This does not seem to be the case when hTRPC1 is expressed in the oocyte (Brereton *et al.*, 2000; see Figs. 2 and 3). In any case, any direct TRPC1 involvement in forming the highly Ca²⁺-selective SOC or Ca²⁺ release-activated current (I_{CRAC}) seems to be reduced by the finding that a novel protein family (i.e., CRAM1 or Orai1) forms I_{CRAC} channels (Peinelt *et al.*, 2006; but see Mori *et al.*, 2002; Huang *et al.*, 2006).

1. Maitotoxin Activates TRPC1 and MscCa

In 1999, xTRPC1 was cloned from *Xenopus* oocytes and shown to be ~90% identical in sequence to the hTRPC1 (Bobanovic *et al.*, 1999). An anti-TRPC1 antibody (T1E3) targeted to an extracellular loop of the predicted protein was generated and shown to recognize an 80-kDa protein. Immunofluorescent staining indicated an irregular “punctuate” expression pattern of xTRPC1 that was uniformly evident over the animal and vegetal hemispheres. Patch clamp studies also indicate that MscCa is uniformly expressed over both hemispheres (Zhang and Hamill, 2000a). This is in contrast to the polarized expression of the ER and the phosphatidylinositol second messenger system, which are more abundantly expressed in the animal hemisphere (Callamaras *et al.*, 1998; Jaconi *et al.*, 1999). These results indicate that neither TRPC1 nor MscCa are tightly coupled to ER internal Ca²⁺ stores and IP₃ signaling. Originally, it was speculated that punctuate TRPC1 expression reflected discrete channel clusters but it could also indicate the channels are localized in microvilli, which make up >50% of the membrane surface (Zhang *et al.*, 2000). In another study, testing the idea that xTRPC1 formed a SOC, Brereton *et al.* (2000) found that antisense oligonucleotides targeting different regions of xTRPC1 sequence did not inhibit IP₃- or thapsigargin-stimulated Ca²⁺ inflow (cf., Tomita *et al.*, 1998). Furthermore, overexpression of hTRPC1 did not enhance the basal or IP₃-stimulated Ca²⁺ inflow (Brereton *et al.*, 2000). However, they did see enhancement of a lysophosphatidic acid (LPA)-stimulated Ca²⁺ influx. Interestingly, LPA also enhances mechanically induced Ca²⁺ influx in a variety of other cells (Ohata *et al.*, 2001). On the basis of the apparent lack of TRPC1-related SOC activity, Brereton *et al.* (2000) speculated that TRPC1 might form the endogenous cation channel activated

by the marine toxin, maitotoxin (MTX). To test this idea, they compared the properties of MTX-activated conductance in normal and in TRPC1-transfected rat liver cells (Brereton *et al.*, 2001), and found that the endogenous MTX-activated conductance displayed properties different from the enhanced MTX-activated conductance expressed in the hTRPC1-transfected cells. In particular, the endogenous conductance showed a higher selectivity for Na^+ over Ca^{2+} and a higher sensitivity to Gd^{3+} block ($K_{50\% \text{ block}} = 1 \mu\text{M}$ vs $3 \mu\text{M}$). These differences were taken to indicate that other endogenous TRPC subunits may normally combine with TRPC1 to form the endogenous MTX-activated conductance, whereas hTRPC1 alone forms the enhanced MTX-activated conductance (Brereton *et al.*, 2001). Unlike with oocytes, it was found that heterologous expression of hTRPC1 in rat liver cells did increase thapsigargin-induced Ca^{2+} inflow.

Evidence from several studies indicates that the oocyte MTX-activated conductance may be mediated by MscCa (Bielfeld-Ackermann *et al.*, 1998; Weber *et al.*, 2000; Diakov *et al.*, 2001). In particular, both display the same cation selectivity, are blocked by 1-mM amiloride and $10\text{-}\mu\text{M}$ Gd^{3+} , are insensitive to flufenamic and niflumic acid, and have a conductance of ~ 25 pS (measured in symmetrical 140-mM K^+ and 2-mM external Ca^{2+}). Because MTX is a highly amphipathic molecule (Escobar *et al.*, 1998), it may activate MscCa by changing bilayer-membrane interactions, as has been proposed for other amphipaths that can activate MS channels in the absence of membrane stretch (Martinac *et al.*, 1990; Kim, 1992; Hamill and McBride, 1996; Casado and Ascher, 1998; Perozo *et al.*, 2002).

2. TRPC1 and Cell Swelling

To directly test whether TRPC1 might be MS, Chen and Barritt (2003) selectively suppressed TRPC1 expression in rat liver cells and measured their response to osmotic cell swelling. Liver cells express MscCa (Bear, 1990) and previous studies had shown that osmotic swelling of epithelial cells activates an MscCa-dependent Ca^{2+} influx that stimulates Ca^{2+} -activated K^+ efflux accompanied by $\text{Cl}^-/\text{H}_2\text{O}$ efflux and RVD (Christensen, 1987). However, in the TRPC1-suppressed liver cells, hypotonic stress caused a greater swelling and faster RVD than observed in control liver cells (Chen and Barritt, 2003). This opposite response may occur because TRPC1 suppression results in a compensatory overexpression of other TRPCs (or redundant RVD mechanisms) that enhance cell swelling and RVD. It should also be recognized that cell swelling does not always activate MscCa. For example, although hypotonic solution activates a robust Ca^{2+} -independent Cl^- conductance in *Xenopus* oocytes that should contribute to RVD, it fails to activate the endogenous MscCa (Ackerman *et al.*, 1994; Zhang and Hamill, 2000a,b).

3. Abnormal TRPC1/MscCa Activity in Duchenne Muscular Dystrophy

Both TRPC1 and MscCa are expressed in skeletal muscle and both have been implicated in the muscular degeneration that occurs in Duchenne muscular dystrophy (DMD). In particular, muscle fibers from the *mdx* mouse (i.e., an animal model of DMD) show an increased vulnerability to stretch-induced membrane wounding (Yeung and Allen, 2004; Allen *et al.*, 2005) that has been linked to elevated $[Ca^{2+}]_i$ levels caused by increased Ca^{2+} leak channel activity (Fong *et al.*, 1990) and/or abnormal MscCa activity (Franco and Lansman, 1990; Chapter 16, Lansman). On the basis of the observation that the channel activity was increased by thapsigargin-induced store depletion, it was proposed that the channel may also be a SOC belonging to the TRPC family (Vandebrouck *et al.*, 2002, see also Hopf *et al.*, 1996). To test this idea, *mdx* and normal muscle were transfected with antisense oligonucleotides designed against the most conserved TRPC regions. The transfected-muscles showed a significant reduction in expression of TRPC1 and TRPC4 but not TRPC6 (all three TRPCs are expressed in normal and *mdx* muscle) and a decrease in the Ca^{2+} leak channel activity. Previous studies indicate that MscCa behaves more like a Ca^{2+} leak channel in *mdx* patches (Franco-Obregon and Lansman, 2002) and in some oocyte patches (Reifarth *et al.*, 1999). It has also been reported that SOC and MscCa in *mdx* muscle display the same single-channel conductance and sensitivity to block by Gd^{3+} , SKF96365, 2APB, and GsMTx4 (Ducret *et al.*, 2006). These studies implicate TRPC1 as being a subunit of both the SOC and MscCa, which given the presence of a dystrophin domain on the C-terminus of TRPC1 (Wes *et al.*, 1995) could explain the shift in gating mode in *mdx* muscle.

4. TRPC1 and Polycystic Kidney Disease

TRPC1 interacts with the putative MS channel TRPP2 when both are heterologously expressed in HEK-293 (Tsiokas *et al.*, 1999), and there is evidence that TRPC1 and TRPP2 may form functional heteromers (Delmas, 2004). TRPP2 is a distant member of the TRP family (polycystin subfamily) and has been shown to form a Ca^{2+} -permeable cation channel that is mutated in the autosomal dominant polycystic kidney disease (ADPKD; Nauli *et al.*, 2003; Nauli and Zhou, 2004; Giamarchi *et al.*, 2006; Chapter 10, Cantiello *et al.*). TRPP2 was originally designated as polycystin kidney disease 2 (PKD2) and shown to combine with PKD1, a membrane protein with a large extracellular N-terminal domain proposed to act as an extracellular sensing antenna for mechanical stimuli. Both TRPP2 and PKD1 are localized in the primary cilium of renal epithelial cells, which is essential for detecting laminar fluid flow (Praetorius and Spring, 2005). However, TRPV4, which is expressed in renal epithelial cells, may also associate with TRPP2

(Giamarchi *et al.*, 2006). It remains to be determined if TRPC1 combines with TRPP2 in renal epithelial cells and whether knock out of TRPC1 and/or TRPV4 blocks fluid flow detection.

5. TRPC1 Is Expressed in Specialized Mechanosensory Nerve Endings

If TRPC1 is a mechanosensory channel, it should be expressed in specialized mechanosensory nerve endings. Glazebrook *et al.* (2005) used immunocytochemical techniques to examine the distribution of TRPC1 and TRPC3–7 in the soma, axons, and sensory terminals of arterial mechanoreceptors, and found that TRPC1, TRPC3, TRPC4, and TRPC5 were expressed in the peripheral axons and the mechanosensory terminals. However, only TRPC1 and TRPC3 extended into the low-threshold mechanosensory complex endings, with TRPC4 and TRPC5 mainly limited to the major branches of the nerve. Although these results are consistent with TRPC1 (and possibly TRPC3) involvement in baroreception, it was concluded that because TRPC1 was not present in all fine terminals that it more likely modulated than directly mediated mechanotransduction. However, it is not clear that all fine endings are capable of transduction. Furthermore, other putative MS proteins (i.e., β and γ ENaC subunits) are expressed in baroreceptor nerve terminals (Drummond *et al.*, 1998) in which case different classes of MS channels (i.e., ENaC and TRPC) may mediate mechanotransduction in different mechanosensory nerves.

6. TRPC1 Involvement in Wound Closure and Cell Migration

The first study to implicate TRPC1 in cell migration was by Moore *et al.* (1998). They proposed that shape changes induced in endothelial cells by activation of TRPC1 were necessary step for angiogenesis. In another study, it was demonstrated that TRPC1 overexpression promoted, while TRPC1 suppression inhibited intestinal cell migration measured by wound closure assay (Rao *et al.* (2006). On the basis of the proposal that MscCa regulates fish keratocyte cell migration (Lee *et al.*, 1999) and the identification of TRPC1 as a MscCa subunit (Maroto *et al.*, 2005), the role of TRPC1 was tested on migration of the highly invasive/metastatic prostate tumor cell line PC-3. TRPC1 activity was shown to be essential for PC-3 cell migration and Gd^{3+} , GsMTx4, anti-TRPC1 antibody, and siRNA-targeting TRPC1 were shown to block PC-3 migration by inhibiting Ca^{2+} dynamics required of cell migration (Maroto *et al.*, 2007, submitted for publication).

7. Reconstitution of TRPC1 as an MS Channel

To identify the protein forming the oocyte MscCa, oocyte membrane proteins were detergent solubilized, fractionated by FPLC, reconstituted

in liposomes, and assayed for MscCa activity using patch recording (Maroto *et al.*, 2005). A specific protein fraction that ran with a conductivity of 16 mS cm^{-1} was shown to reconstitute the highest MscCa activity and silver-stained gels indicated it displayed the highest abundance of 80-kDa protein. On the basis of previous studies that identified xTRPC1 and hTRPC1 as forming an 80-kDa protein when expressed oocytes (Bobanovic *et al.*, 1999; Brereton *et al.*, 2000), immunological methods were used to demonstrate that TRPC1 was present in the MscCa active fraction. Furthermore, heterologous expression of the hTRPC1 was shown to greatly increase the MscCa activity expressed in the transfected oocyte, whereas TRPC1-antisense reduced the endogenous MscCa activity (Maroto *et al.*, 2005). Figure 2 compares MscCa activity in cell-attached patches on a control oocyte (Fig. 2A) and an oocyte that had been injected with hTRPC1 (Fig. 2B). Despite the almost tenfold increase in current density in the TRPC1-injected oocyte, channel activation and deactivation kinetics in the two patches were similar. However, in some patches, even on the same oocyte, the kinetics of the TRPC1-dependent channels show delayed activation and deactivation kinetics. An example of the slow kinetics is illustrated for a patch that was formed on an oocyte that had been injected with TRPC1 with enhanced green fluorescence protein (eGFP) attached to the C-terminus. Figure 3 shows confocal fluorescence images of the oocyte at low magnification and at high magnification indicating eGFP-TRPC1 concentrated in the surface membrane (Fig. 3).

Figure 4 compares the patch response on a control oocyte and the slow kinetics response of a patch formed on the oocyte displayed in Fig. 3. The basis for the heterogeneity in kinetics of TRPC1 channels may reflect local differences in the underlying CSK and/or bilayer or even the MscCa subunit composition that occurs with TRPC1 overexpression. Maroto *et al.* (2005) also demonstrated that hTRPC1 expression in CHO cells results in increased MscCa activity, consistent with an approximately fivefold greater increase in channel density. The presence of endogenous MscCa activity is consistent with previous reports that indicate CHO cells express TRPC1 along with TRPC2–6 (Vaca and Sampier, 2002). Although the above results provide compelling evidence that TRPC1 is a structural component on the MscCa, the current increase in TRPC1-transfected oocytes and CHO cells is relatively low compared with that achieved by overexpression of other channel types. This may be because endogenous TRPC1 needs to combine with endogenous TRPCs or other ancillary proteins. On the other hand, the ability to reconstitute MscCa activity following 5000-fold protein to lipid dilution would seem to argue against the requirement of at least ancillary proteins that are not firmly attached to the channel complex.

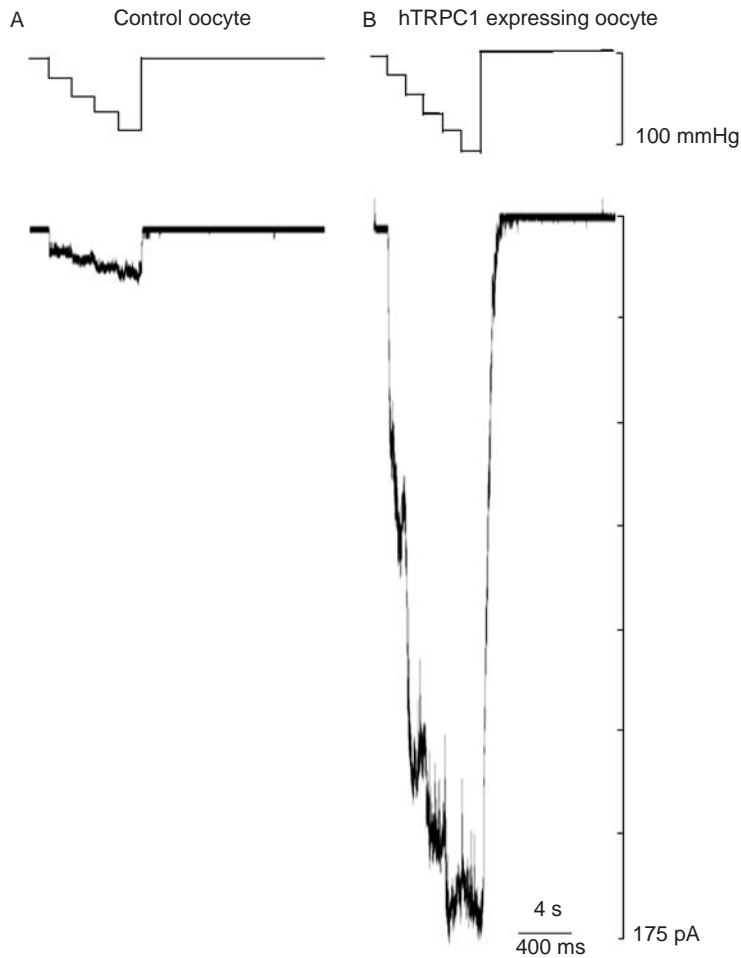


FIGURE 2 MS current activity measured in a control and an hTRPC1-expressing oocyte. (A) Stepwise increase in suction (top trace) applied to a cell-attached patch formed on a control oocyte (i.e., that was injected with 50 nl of water 4 days earlier) induced a current of 12 pA. (B) Similar to A except that the patch was formed on an hTRPC1-expressing oocyte (i.e., injected 4 days earlier with 50 nl of TRPC1 transcripts). In this case, the peak current produced was 175 pA. Examination of the residual channels immediately after the steps indicates the same single-channel currents of ~ 2 pA. Both recordings were made at a patch potential of -50 mV. (From Maroto *et al.*, 2005).

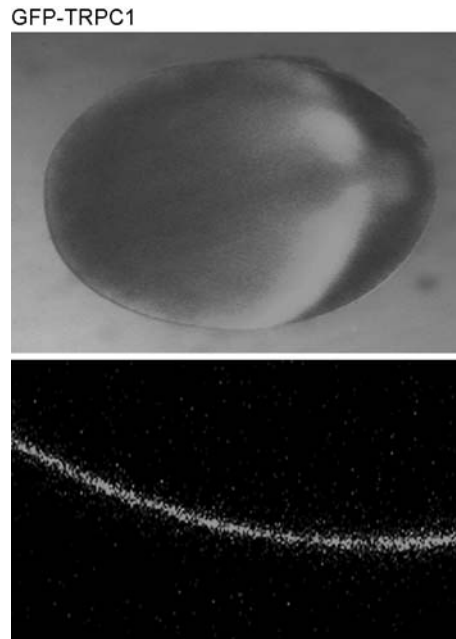


FIGURE 3 Fluorescence images of an oocyte that had been injected 3 days earlier with mRNA construct encoding enhanced green fluorescence protein attached to the C-terminus of *Trpc1*. Upper panel shows whole oocyte. Lower panel shows confocal images focussed on the oocyte edge. (See Color Insert.)

B. TRPC2

At present there is no evidence, direct or indirect, to indicate TRPC2 forms an MS channel. The current view is that it may function as a ROC or a SOC depending on cell type (Vannier *et al.*, 1999; Gailly and Colson-Van Schoor, 2001; Chu *et al.*, 2004; Zufall *et al.*, 2005). For example, TRPC2 has been implicated in pheromone detection in the rodent vomeronasal organ (VNO; Liman *et al.*, 1999) because TRPC2^{-/-} mice lack gender discrimination (Zufall *et al.*, 2005). Because a DAG-activated channel in VNO neurons is downregulated in TRPC2^{-/-} mice and TRPC2 is localized in the sensory microvilli that lack Ca²⁺ stores, it seems that TRPC2 functions as a ROC rather than a SOC at least in VNO neurons (Spehr *et al.*, 2002; Zufall *et al.*, 2005). However, in erythroblasts, and possibly sperm, TRPC2 has been reported to be activated by store depletion. In both cell types, the long splice

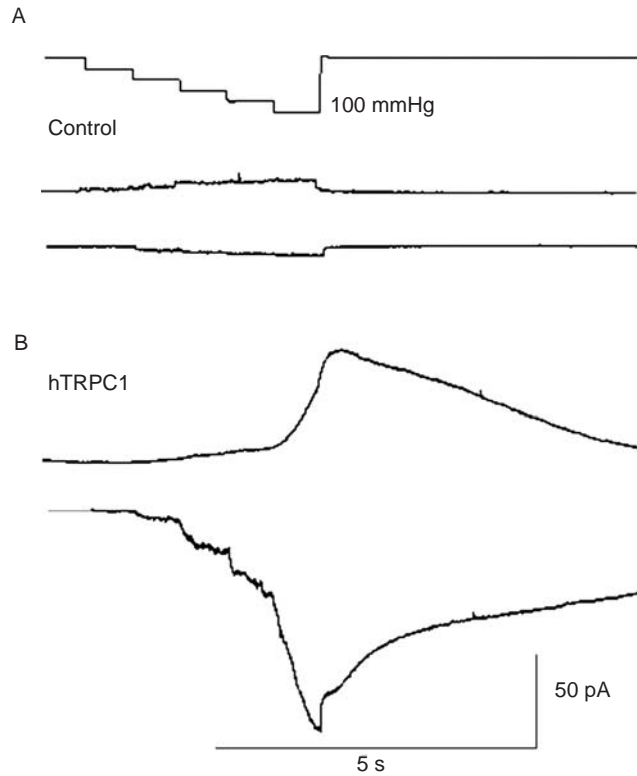


FIGURE 4 Oocyte patches formed on TRPC1-expressing oocytes can show slow turn-on and delayed turn-off in addition to the greatly enhanced currents. (A) Cell-attached patch on a control oocyte showing step responses similar to Fig. 2A. (B) Cell-attached patch on the oocyte shown in Fig. 3 that had been injected with hTRPC1 transcripts. In this case, the pressure-stimulated currents were not only much larger than the wild-type responses but also failed to saturate and exhibited a pronounced delay in both its turn-on and turn-off with the pressure steps. Fast responses similar to Fig. 2B were also seen on this oocyte.

variants of TRPC2 were detected (Yildrin *et al.*, 2003), whereas VNO neurons express the short splice variant (Hofmann *et al.*, 2000; Chu *et al.*, 2002). In sperm, TRPC2 may participate in the acrosome reaction-based inhibition by a TRPC2 antibody *in vitro* (Jungnickel *et al.*, 2001). However, TRPC2^{-/-} mice display normal fertility therefore casting doubt on this role (Stambouljian *et al.*, 2005). In hematopoiesis, erythropoietin modulates Ca²⁺ influx via TRPC2 in possible combination with TRPC6 (Chu *et al.*, 2002, 2004).

C. TRPC3

As with TRPC2, there is no evidence yet for TRPC3 mechanosensitivity. However, TRPC3 does colocalize with TRPC1 in specialized mechanosensory nerve endings, indicating that both may combine to form an MS channel (Glazebrook *et al.*, 2005). The growing consensus is that TRPC3 can contribute to both SOC and ROC channels depending on expression levels (Zitt *et al.*, 1997; Hofmann *et al.*, 1999; Hurst *et al.*, 1998; Kamouchi *et al.*, 1999; Trebak *et al.*, 2002; Putney *et al.*, 2004; Vazquez *et al.*, 2005; Groschner and Rosker, 2005; Liu *et al.*, 2005; Zagranichnaya *et al.*, 2005; Kawasaki *et al.*, 2006). Suppression of TRPC3 in cerebral arterial smooth muscle while suppressing pyridine receptor-induced depolarization does not alter the pressure-increased depolarization and contraction, which appears to be dependent on TRPC6 (Reading *et al.*, 2005). On the other hand, TRPC3 activation appears to depend upon Src kinase that may be MS (Vazquez *et al.*, 2004b) and like TRPC6 is directly activated by OAG (Hofmann *et al.*, 1999).

D. TRPC4

There is disagreement on whether TRPC4 functions as a SOC and/or ROC (Philipp *et al.*, 1996; Tomita *et al.*, 1998; McKay *et al.*, 2000; Schaefer *et al.*, 2000; Plant and Schaefer, 2005). TRPC4 has been suggested to form a ROC activated by AA (Wu *et al.*, 2002; Zagranichnaya *et al.*, 2005). In particular, using siRNA and antisense strategies to reduce endogenous TRPC4 expression, TRPC4 was shown to be required for the OAG-induced and receptor-operated Ca^{2+} entry as well as the AA-induced Ca^{2+} oscillations but not for SOC function. This AA activation may have implications for the mechanosensitivity of TRPC4 since AA has been shown to activate a variety of MS channels in the absence of applied stretch where it appears to act by directly altering mechanical properties of the bilayer surrounding the channel (Kim, 1992; Hamill and McBride, 1996; Casado and Ascher, 1998; Patel *et al.*, 2001). Studies of TRPC4^{-/-} mice indicate TRPC4 is an essential determinant of endothelial vascular tone and endothelial permeability as well as neurotransmitter release from central neurons (reviewed by Freichel *et al.*, 2004).

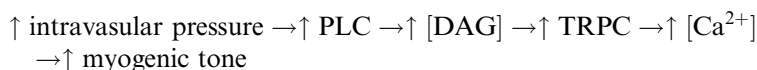
E. TRPC6

The general consensus is that TRPC6 forms a ROC that is activated by DAG in a membrane-delimited fashion and is insensitive to activation by IP₃ and store depletion (Boulay *et al.*, 1997; Hofmann *et al.*, 1999; Estacion

et al., 2004; Zagranichnaya *et al.*, 2005; Zhang *et al.*, 2006). Although TRPC6 is a member of the TRPC3/6/7 subfamily, it shows distinct functional and structural properties. Functionally, while TRPC6 only forms a ROC, TRPC3 and TRPC7 appear capable of participating in forming both ROCs and SOC (Zagranichnaya *et al.*, 2005); structurally, whereas TRPC6 carries two extracellular glycosylation sites, TRPC3 carries only one (Dietrich *et al.*, 2003). Furthermore, exogenously expressed TRPC6 shows low basal activity compared with TRPC3 and elimination of the extra glycosylation site that is missing in TRPC3 transforms TRPC6 into a constitutively active TRPC3-like channel. Conversely, engineering of an additional glycosylation site in TRPC3 markedly reduces TRPC3 basal activity.

1. TRPC6 as a Regulator of Myogenic Tone

TRPC6 is proposed to mediate the depolarization and constriction of small arteries and arterioles in response to adrenergic stimulation (Inoue *et al.*, 2001; Jung *et al.*, 2002; Inoue *et al.*, 2006), and elevation of intravascular pressure consistent with TRPC6 forming a MOC as well as a ROC (Welsh *et al.*, 2000, 2002). The cationic current activated by pressure in vascular smooth muscle is suppressed by antisense-DNA to TRPC6 (Welsh *et al.*, 2000). Furthermore, because the cation entry was stimulated by OAG and inhibited by PLC inhibitor (Park *et al.*, 2003), it was proposed that TRPC6 forms an MS channel that is activated indirectly by pressure according to the pathway:



In this scheme, it is PLC rather than TRPC that is MS. This would imply that since all TRPCs are coupled to PLC-dependent receptors, they may all display mechanosensitivity. However, while there are reports that PLC can be mechanically stimulated independent of external Ca^{2+} (Mitchell *et al.*, 1997; Rosales *et al.*, 1997; Moore *et al.*, 2002), there are also studies that indicate the mechanosensitivity of PLC derives from stimulation by Ca^{2+} influx via MscCa (Matsumoto *et al.*, 1995; Ryan *et al.*, 2000; Ruwhof *et al.*, 2001). In this case, it becomes important to demonstrate that TRPC6 can be mechanically activated in the absence of external Ca^{2+} (e.g., using Ba^{2+}). There is other evidence to indicate TRPC6 may be coupled to other MS enzymes. For example, TRPC6 is similar to TRPV4 in that it is activated by 20-hydroxyeicosatetraenoic acid (20-HETE), which is the dominant AA metabolite produced by cytochrome P-450 w-hydroxylase enzymes (Basora *et al.*, 2003). TRPC6 may also be activated by Src family protein tyrosine kinase (PTK)-mediated tyrosine phosphorylation (Hisatsune *et al.*, 2004). Indeed, PP2, a specific inhibitor of Src

PTKs, abolishes TRPC6 (and TRPC3) activation and strongly inhibits OAG-induced Ca^{2+} entry (Soboloff *et al.*, 2005). OAG may operate solely through TRPC6 homomers, the cation of vasopressin (VP) may also include the OAG-insensitive TRPC heteromers (e.g., TRPC1 and TRPC6). A further complication is that DAG-dependent activation of PKC appears to stimulate the myogenic channels based on their block by the PKC inhibitor chelerythrine (Sligh *et al.*, 2002), whereas PKC activation seems to inhibit TRPC6 channels, which would seem more consistent with direct activation by DAG/OAG (Soboloff *et al.*, 2005).

Despite the above evidence implicating TRPC6 as the “myogenic” channel, TRPC6-deficient mice show enhanced rather than reduced myotonic tone and increased rather than reduced responsiveness to constrictor agonist in small arteries. These effects result in both a higher elevated mean arterial blood pressure and a shift in the onset of the myogenic tone toward lower intravascular pressures, again opposite to what would be expected if TRPC6 was critical for myoconstriction (Dietrich *et al.*, 2005). Furthermore, isolated smooth muscle from TRPC6^{-/-} mice show increased basal cation entry and more depolarized resting potentials, but both effects are blocked if the muscles are also transfected with siRNA-targeting TRPC3. On the basis of this last observation, it was suggested that constitutively active TRPC3 channels are upregulated in TRPC6^{-/-} mice. However, the TRPC3 subunits are unable to functionally replace the lost TRPC6 function that involves suppression of high basal TRPC3 activity (i.e., the TRPC3/TRPC6 heteromer is a more tightly regulated ROC and/or MOC). In summary, although evidence indicates TRPC6 may be a pressure or stretch-sensitive channel and contribute to MOC, the TRPC6 knockout mouse indicates a phenotype that cannot be explained if TRPC6 alone forms the vasoconstrictor channel. It may also be relevant that another study could find no evidence that Gd^{3+} -sensitive MscCa contributes to myogenic tone in isolated arterioles from rat skeletal muscle (Bakker *et al.*, 1999).

2. TRPC6 as a Regulator of the Kidney Slit Diaphragm

Autosomal dominant focal segmental glomerulosclerosis (FSGS) is a kidney disease that leads to progressive renal kidney failure characterized by leakage of plasma proteins like albumin into the urine (proteinuria). Mutations in TRPC6 were associated with familial FSGS and implicated in aberrant Ca^{2+} signaling that leads to podocyte injury (Reiser *et al.*, 2005; Winn *et al.*, 2005). Furthermore, two of the mutants were demonstrated to be gain-of-function mutations that produce larger ROCs than the wild-type TRPC6 expressed in HEK-293 cells. Ultrafiltration of plasma by the renal glomeruli is mediated mainly by the podocyte, which is an epithelial cell that

lies external to the glomerular basement membrane (GBM) and lines the outer endothelium of the capillary tuft located inside the Bowman's capsule. The podocyte covers the GBM and forms interdigitating foot processes that are connected by slit diaphragms—ultra-thin membrane structures that form at the center of the slit a zipper-like structure with pores smaller than albumin (Kriz, 2005; Tryggvason and Wartiovarara, 2005). The podocyte-specific proteins, nephrin and podocin, are localized in the slit diaphragm and the extracellular domains of nephrin molecules of neighboring foot processes interact to form the zipper structure. Podocin, a member of the stomatin family, is a scaffolding protein that accumulates in lipid rafts and interacts with the cytoplasmic domain of nephrin (Durvasula and Shankland, 2006). Both nephrin and podocin have been shown to be mutated in different familial forms of FSGS. Furthermore, TRPC6 interacts with both nephrin and podocin and a nephrin deficiency in mice leads to overexpression and mislocalization of TRPC6 in podocyte as well as disruption of the slit diaphragm (Reiser *et al.*, 2005). Mechanical forces play an important role in ultrafiltration both in terms of the high transmural distending forces arising from the capillary perfusion pressure as well as the intrinsic forces generated by the contractile actin network in the foot process that control, in a Ca^{2+} -dependent manner, the width of the filtration slits. As a consequence, TRPC6 may act as the central signaling component mediating pressure-induced constriction at the slit. In summary, two quite diverse physiological functions, myogenic tone and renal ultrafiltration, implicate TRPC6 as an MS channel. However, whether TRPC6 acts as a direct mechanosensor as in the case of TRPC1 or is indirectly MS like TRPV4 remains to be determined.

IX. CONCLUSIONS

At least three basic mechanisms referred to as “bilayer,” “conformational coupling,” and “enzymatic” may confer mechanosensitivity on TRPCs. The bilayer mechanism should operate if the TRPC, in shifting between closed and open states, undergoes a change in its membrane-occupied area, thickness, and/or cross-sectional shape. Any one of these changes would confer mechanosensitivity on the channel. A bilayer mechanism may also underlie the ability of lipidic second messengers (e.g., DAG/OAG, AA, lysophospholipid and 5',6'-EET) to directly activate TRPCs by inserting in the bilayer to alter its local bilayer packing, curvature, and/or the lateral pressure profile. The only unequivocal way to demonstrate that a bilayer mechanism operates is to show that stretch sensitivity is retained when the purified channel protein is reconstituted in liposomes. At this stage, one can go onto measure

channel activity as a function of changing bilayer thickness (i.e., by using phospholipids with different acyl length chains) and local curvature/pressure profile (i.e., by using lysophospholipids with different shapes; Perozo *et al.*, 2002; Martinac, 2007; Markin and Sachs, 2007; Powl and Lee, 2007).

The second mechanism involves conformational coupling (CC) that has been evoked to account for TRPC sensitivity to depletion of internal Ca^{2+} stores. CC was originally used to explain excitation–contraction (E–C) coupling, involving the physical coupling between L-type Ca^{2+} channel (i.e., dihydropyridine receptors, DHPR) in the plasma membrane and ryanodine receptors (RyR1) that release Ca^{2+} from the sarcoplasmic reticulum (SR; Protasi, 2002). Subsequently, a retrograde form of CC was discovered between the same two proteins that regulate the organization of the DHPR into tetrads and the magnitude of the Ca^{2+} current carried by DHPR (Wang *et al.*, 2001; Paolini *et al.*, 2004; Yin *et al.*, 2005). Another form of CC was demonstrated associated with physiological stimuli that do not deplete Ca^{2+} stores yet activate Ca^{2+} entry through channels referred to as excitation-coupled Ca^{2+} entry channels to distinguish them from SOC (Cherednichenko *et al.*, 2004). Interestingly, RyR1 is functionally coupled to both TRPC1-dependent SOC and TRPC3-dependent SR Ca^{2+} release (Sampieri *et al.*, 2005; Lee *et al.*, 2006).

A key issue for all forms of CC is whether the direct physical link that conveys mechanical conformational energy from one protein to another can also act as a pathway to either focus applied mechanical forces on the channel or alternatively constrain the channel from responding to mechanical forces generated within the bilayer. Another possibility is that reorganization or clustering of the resident ER protein (i.e., STIM) that senses Ca^{2+} stores may alter channel mechanosensitivity by increasing the strength of CC coupling (Kwan *et al.*, 2003).

Some insights into these possibilities can be provided by the process of “membrane blebbing,” which involves decoupling of the plasma membrane from the underlying CSK and has been shown to either increase or decrease the mechanosensitivity of MS channels depending on the channel (Hamill and McBride, 1997; Hamill, 2006). Since membrane blebbing would also be expected to disrupt any dynamic interactions between TRPC and scaffolding proteins, it should alter TRPC function. In one case it has been reported that Ca^{2+} store depletion carried out after but not before formation of a tight seal is effective in blocking the activation of SOC channels in the frog oocyte patches (Yao *et al.*, 1999). Presumably, this occurs because the sealing process physically decouples the channels from ER proteins that sense internal Ca^{2+} stores. Tight seal formation using strong suction can also reduce MscCa mechanosensitivity and gating kinetics possibly by a related mechanism (Hamill and McBride, 1992). On the other hand, it has been reported that

I_{CRAC} is retained following cell “ballooning” (i.e., a form of reversible membrane blebbing), indicating that the coupling between the channel and the Ca^{2+} sensor STIM may be relatively resistant to decoupling (Bakowski *et al.*, 2001). In any case, in order to directly demonstrate a role for CC in mechanosensitivity, one needs to show that stretch sensitivity can be altered in mutants in which TRPC–ancillary protein interactions are disrupted (Section VII.D).

The third mechanism of mechanosensitivity relates to functional coupling between TRPCs and MS enzymes. Apart from the PLA_2 and Src that are MS and have been implicated in conferring mechanosensitivity on TRPV4 (Vriens *et al.*, 2004; Cohen, 2005a,b), there is growing evidence that PLC is also MS with reports indicating that mechanosensitivity is either dependent on external Ca^{2+} and Ca^{2+} influx (Matsumoto *et al.*, 1995; Ryan *et al.*, 2000; Ruwhof *et al.*, 2001; Alexander *et al.*, 2004) or Ca^{2+} independent (Mitchell *et al.*, 1997; Rosales *et al.*, 1997; Moore *et al.*, 2002). In either case, these studies indicate that mechanical forces transduced by MscCa and/or by MS enzymes may modulate the gating of all TRP channels. It remains to be determined what are the physiological and/or pathological effects of this MS modulation? The methods discussed in this chapter, including the applications of pressure steps to measure the kinetics of MS enzyme–channel coupling and the use of membrane protein liposome reconstitution for identifying specific protein–lipid interactions, should play an increasing role in understanding the importance of the different MS mechanisms underlying TRPC functions.

Note Added in Proof

Spasova, M. A., Hewavitharana, T., Xu, W., Soboloff, J., and Gill, D. L. (*Proc. Natl. Acad. Sci. USA* **103**, 16586–16591) have reported that overexpression of hTRPC6 in mammalian cells results in increased OAG- and swelling-activated whole cell currents and increased stretch-activated channel activity in inside-out patches. The TRPC6 activity was blocked by GsmTX4 but was insensitive to block by the PLC inhibitor U73122 (c.f., Park *et al.*, 2003). Furthermore, they found that the long delays associated with stretch activation of TRPC6 channels could be reduced by treatment of cells with cytochalasin D. These results are consistent with TRPC6 being directly MS and a common bilayer mechanism underlying OAG- and stretch-activation of TRPC6.

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CHAPTER 17

MscCa Regulation of Tumor Cell Migration and Metastasis

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I. OVERVIEW

The acquisition of cell motility is a required step in order for a cancer cell to migrate from the primary tumor and spread to secondary sites (metastasis). For this reason, blocking tumor cell migration is considered a promising approach for preventing the spread of cancer. However, cancer cells like normal cells can migrate by several different modes referred to as

“amoeboid,” “mesenchymal,” and “collective cell.” Furthermore, under appropriate conditions a single cell can switch between modes. A consequence of this plasticity is that a tumor cell may be able to avoid the effects of an agent that targets only one mode by switching modes. Therefore, a preferred strategy would be to target mechanisms that are shared by all modes. Here we review the evidence that Ca^{2+} influx via the mechanosensitive Ca^{2+} -permeable channel (MscCa) is a critical regulator of all modes of cell migration and therefore represents a very good therapeutic target to block metastasis.

II. INTRODUCTION

Cancer is a multistep process that results in a normal cell, often an epithelial cell lining a gland, duct, or organ surface, undergoing abnormally increased multiplication to produce a localized primary tumor that with time invades and spreads (metastasizes) to surrounding tissues and eventually causes death. However, in order for a tumor to metastasize, the tumor cell must migrate from the primary tumor, pass through blood vessels, penetrate into the secondary tumor site, and migrate through the tissue to establish a metastasis. Therefore, the acquisition of cell motility is a necessary although not a sufficient step for tumor invasion and metastasis, which also require the additional steps of barrier matrix breakdown, tumor cell adherence, growth, and angiogenesis at the secondary sites. Nevertheless, because metastasis will only be achieved if the tumor cell completes every step in the metastatic cascade, identifying the most sensitive and susceptible step that regulates tumor cell migration should provide a promising target to block metastasis (Grimstad, 1987; Stracke *et al.*, 1991; Kassis *et al.*, 2001).

There are currently two models used to explain tumor progression to the metastatic disease. One is the traditional “multi-hit” genetic model that proposes a sequence of mutations that triggers the various stages of cancer (e.g., initiation, promotion) with the final mutation(s) promoting increased tumor cell invasiveness and metastasis (Emmelot and Scherer, 1977; Cahill *et al.*, 2000; Hanahan and Weinberg, 2000; Zhou *et al.*, 2005). Evidence supporting this model includes the existence of several stable human tumor cell lines that demonstrate high invasiveness when implanted in animals (Kaighn *et al.*, 1979; Sung *et al.*, 1998), and the recent discovery that many primary tumor cells already express a genetic signature that predicts their metastatic potential (Ramaswamy *et al.*, 2003; Varambally *et al.*, 2005). The second model is an epigenetic one based on the discovery that growth factors that trigger the epithelial–mesenchymal transition (EMT), in which nonmotile epithelial cells are converted into motile mesenchymal cells (e.g., during

normal embryogenesis and wound healing), are also released by stromal cells surrounding the tumor and promote increased tumor cell invasiveness and metastasis (Thiery, 2002; Thompson and Newgreen, 2005; but see Tarin, 2005). Specific cancers may utilize one or a combination of the two mechanisms since the mechanisms are not exclusive (e.g., one aspect of the metastatic genetic signature may include the potential to undergo EMT). In any case, the regulatory molecules involved in transforming a tumor cell from a nonmotile to a motile phenotype need to be identified. In this chapter we focus on the role of the MscCa, which is identified as a member of the transient receptor potential channel family (Maroto *et al.*, 2005; Saimi *et al.*, 2007) and shown to be essential for prostate tumor cell migration (Maroto *et al.*, 2007). Because MscCa is expressed by both nonmotile and motile cells, we review the evidence for the idea that changes in MscCa properties triggered by events associated with cancer progression may contribute to increased tumor invasiveness and metastasis.

III. DIFFERENT MODES OF MIGRATION

Normal cells and tumor cells move according to one of three major modes of migration referred to as “amoeboid,” “mesenchymal,” and “collective cell.” Furthermore, under specific circumstances a single cell can switch between these modes (Friedl and Wolf, 2003; Sahai and Marshall, 2003; Friedl, 2004; Wolf and Friedl, 2006). Because of this plasticity, a tumor cell may be able to avoid the effects of an agent that blocks only one migratory mode by switching to another mode. Therefore, a preferred strategy would be to identify and target molecular mechanisms that are shared by all modes. With this in mind, we consider the different modes of migration, their similarities and differences, and in particular their possible common dependence on Ca^{2+} influx via MscCa.

A. Amoeboid Migration

Amoeboid movement is expressed by a variety of invertebrate and vertebrate cells, but has been the most intensely studied in the amoeba *Dictyostelium discoideum*. This cell displays an ellipsoidal profile with either a monopodal or polypodal form, and undergoes a rapid (e.g., $>20 \mu\text{m}/\text{min}$) gliding movement that involves repetitive cycles of protrusion and contraction with little adhesiveness to the substrate. This lack of adhesiveness is consistent with the absence of integrin expression by the amoeba (Friedl, 2004). The amoeba uses two mechanically distinct mechanisms to push itself

forward (Yoshida and Soldati, 2006) a filopodia–lamellipodia mechanism that depends on actin polymerization and a bleb mechanism in which a local region of membrane where the cortical-CSK has been disrupted is pushed outward by cytoplasmic pressure generated by myosin II. Both protrusion mechanisms involve significant mechanical distortions of the membrane at the front of the cell that could activate MscCa to provide feedback (via Ca^{2+} influx and/or membrane polarization) between the force-generating mechanisms and resultant membrane distortions.

Neutrophils, eosinophils, lymphocytes, stem cells, and specific tumor cells associated with leukemia, lymphoma, and small cell lung carcinoma also display amoeboid movement. Furthermore, specific cell types that display a mesenchymal mode of migration when crawling on a two-dimensional (2D) substrate can switch to an amoeboid mode when migrating through a 3D substrate (Friedl, 2004). Vertebrate cells undergoing amoeboid migration also display both blebbing and filopodia–lamellipodia mechanisms of forward protrusion (Sahai and Marshall, 2003; Blaser *et al.*, 2006). Fish and amphibian keratocytes may represent a hybrid form of amoeboid/mesenchymal locomotion because they normally show a smooth gliding movement but also express a broad flat lamellipodium. Furthermore, when they become stuck on their substrate they tend to pull out a rear tether and display a more discontinuous “mesenchymal-like” locomotion (Lee *et al.*, 1999). Interestingly, an amoeba can be induced to develop a broad lamellipodium and undergo keratocyte-like migration by knocking out a gene that regulates the amoeba’s aggregation process (Asano *et al.*, 2004). However, a double knockout of myosin II and the aggregation gene does not block keratocyte-like migration, indicating that myosin II may be dispensable for this mode of movement.

B. Mesenchymal Migration

Mesenchymal movement is displayed by fibroblasts, neurons, smooth muscle, and endothelial cells, as well as by specific cancer cells from epithelial tumors, gliomas, and sarcomas. In this mode, the cell typically displays a highly polarized morphology with a front lamellipodium, immediately behind which is the lamella, followed by the cell body with the nucleus, and usually ending with a rear tail or tether. Compared with the smooth, gliding amoeboid movement, mesenchymal migration is relatively discontinuous and slower ($<1 \mu\text{m}/\text{min}$) because of its greater adhesiveness and strong dependence on integrin engagement and disengagement from the substrate. Mesenchymal migration can be divided into five steps involving: (1) forward protrusion of the cell’s leading edge, (2) formation of adhesions at the front

of the cell with the extracellular matrix (“gripping”), (3) pulling against the ECM via the cell adhesions as the myosin–cytoskeleton (CSK) contracts and exerts traction force against the substrate, (4) progressive stretching of the cell as the traction force develops at the cell front and pulls against the cell rear, and (5) finally, detachment of the rear adhesions from the ECM allowing net cell displacement and relaxation of membrane stretch (Lauffenburger and Horwitz, 1996; Sheetz *et al.*, 1999; Ridley *et al.*, 2003). The important aspect of this mode of migration in relation to MscCa is that the membrane bilayer of the whole cell will tend to experience a slow ramp of increasing tension for as long as the rate of forward protrusion exceeds the rate of rear retraction (Lee *et al.*, 1999; Maroto *et al.*, 2007).

C. Collective Cell Migration

In the collective cell mode of migration, the cells are connected by cell junctions formed by cadherins and integrins, and move in a mass with the motile cells at the leading invasive edge generating the adhesion and traction forces (likely via the mesenchymal mode) that tend to pull the rear nonmotile tumor cells along passively. This pattern of migration represents the predominate migration mode for most epithelial cancers *in situ*, and provides the advantage of increased heterogeneity by allowing nonmotile, proliferating cells along with motile path-finding cells to invade the new tissues (Friedl and Wolf, 2003; Wolf and Friedl, 2006).

D. Mechanisms for Switching Migration Modes

Cells that normally express mesenchymal and/or collective cell migration can be converted to the amoeboid mode by reducing the effectiveness of integrin-ECM adhesion (i.e., with integrin-blocking antibodies or arginine-glycine aspartate (RGD) peptides that compete for integrin-ECM-binding sites), by blocking matrix proteases, or by stimulating the Rho-associated serine/threonine kinase (ROCK) that increases cortical contraction, thereby promoting cell rounding and forward protrusion by membrane blebbing (Friedl, 2004). With this switch, the cell becomes more deformable due to its lack of adhesiveness and can now squeeze between matrix barriers. This lessens the dependence on the actions of matrix-degrading metalloproteinases and increases resistance to metalloproteinase inhibitors. The weakened dependence on integrin adhesion also results in a loss of dependence on calpain proteolytic cleavage important for integrin-linked adhesion turnover (Carragher *et al.*, 2005). In neutrophils, rear integrins tend to be endocytosed

rather than dissembled by calpain activity, and in contrast to mesenchymal cells, inhibition of calpain actually promotes, rather than inhibits, migration by enhancing cell protrusion and cell spreading (Lokuta *et al.*, 2003). On the other hand, amoeboid movement retains a strong dependence on myosin II contractility as indicated by increased sensitivity to ROCK inhibition (Sahai and Marshall, 2003). Since that both calpain and myosin II are Ca^{2+} sensitive, one would expect that both modes of migration would display Ca^{2+} dependence. Another mechanism that appears to promote mode switching relates to the relocation of cavin-1 (Cav-1), a lipid raft-associated protein that colocalizes with MscCa/TRPC1 (Lockwich *et al.*, 2000; Brazier *et al.*, 2003; Maroto *et al.*, 2005). For example, when endothelial cells switch from migration in a 2D to a 3D matrix there is a redistribution of Cav-1, and possibly MscCa, from the back to the front of the cell (Parat *et al.*, 2003). As described below, this shift would be consistent with intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) transients being initiated in the front of the amoeboid like neutrophils (Kindzelskii *et al.*, 2004) but in the rear of mesenchymal-like cells (Maroto *et al.*, 2007).

IV. Ca^{2+} DEPENDENCE OF CELL MIGRATION

Although a variety of signaling pathways may regulate cell migration, Ca^{2+} signaling has always been considered a significant player because many of the effector molecules that mediate migration are Ca^{2+} sensitive, including myosin light chain kinase (i.e., that regulates myosin II), calpain, gelsolin, α -actinin, and phosphatase (calcineurin) and integrins (Hendey and Maxfield, 1993; Arora and McCulloch, 1996; Eddy *et al.*, 2000; Mamoune *et al.*, 2003; Franco and Huttenlocher, 2005). The Ca^{2+} regulatory role has been reinforced by the finding that a variety of Ca^{2+} transport proteins including pumps, exchangers, and various gated Ca^{2+} channels can modulate cell migration (Dreval *et al.*, 2005).

A. Measuring $[\text{Ca}^{2+}]_i$

The most convenient and common method used to measure $[\text{Ca}^{2+}]_i$ involves using fluorescent microscopy and Ca^{2+} -sensitive fluorescent dyes like fura-2 and its membrane permeable form fura-2 AM (Grynkiewicz *et al.*, 1985). The main advantage of the approach is that changes in $[\text{Ca}^{2+}]_i$ can be monitored while simultaneously measuring cell migration (i.e., by time-lapse videomicroscopy). As a consequence, one can relate specific spatio-temporal changes in $[\text{Ca}^{2+}]_i$ to specific events occurring during migration. However,

there are also some practical limitations associated with the method, including the difficulty of detecting local vs global $[Ca^{2+}]_i$ changes and the possibility of compartmentalization of the dyes in organelles. The first limitation has been somewhat overcome by recent technical developments that includes the use of total internal reflectance fluorescence microscopy that offers added spatial resolution to allow detection of single-channel $[Ca^{2+}]_i$ fluctuations at the ventral membrane surface adhering with the glass surface (Demuro and Parker, 2005). In addition, the development of Ca^{2+} -sensor “cameleons” that operate by fluorescence energy transfer and can be targeted to the plasma membrane or the ER can be used to measure $[Ca^{2+}]_i$ changes in these membrane microdomains (Miyawaki *et al.*, 1997; Isshiki *et al.*, 2002). In the case of fura-2 compartmentalization, there are discrepant views on its occurrence and significance. For example, one group has proposed that the apparent $[Ca^{2+}]_i$ gradient seen in T lymphocytes is due to fura-2 accumulation in mitochondria (Quintana and Hoth, 2004), whereas another group found that the $[Ca^{2+}]_i$ gradient seen in fibroblasts was not associated with mitochondria but instead colocalized with the Golgi apparatus in the perinuclear region (Wahl *et al.*, 1992). A further complication is that mitochondria are motile, and their motility varies inversely with $[Ca^{2+}]_i$ so that they move fastest in lower $[Ca^{2+}]_i$ (100–300 nM) but stop movement in higher $[Ca^{2+}]_i$ (i.e., 1 μ M) (Yi *et al.*, 2004). As a consequence, one would expect mitochondria to migrate up a $[Ca^{2+}]_i$ gradient and accumulate in regions of highest $[Ca^{2+}]_i$ where they may function as Ca^{2+} buffers and/or prevent the spread of local $[Ca^{2+}]_i$ transients (Tinel *et al.*, 1999; Yi *et al.*, 2004; Levina and Lew, 2006). However, in apparent contradiction of this idea, mitochondria accumulate in the lamellipodium of migrating fibroblasts and prostate tumor cells (DeBiasio *et al.*, 1987; Maroto *et al.*, 2007), and yet these cells develop a global $[Ca^{2+}]_i$ gradient that increases from front to back of the cell (Hahn *et al.*, 1992; Matoto *et al.*, 2007). The stimulus that promotes this accumulation remains unclear but could involve the added requirement for ATP and/or an elevated $[Ca^{2+}]_i$ in membrane subdomains of the lamellipodium. In any case, it would appear that compartmentalization of fura-2 dye cannot alone explain the sustained, and in some cases rapidly reversible, $[Ca^{2+}]_i$ gradients seen in a variety of migrating cells (see Section IV.E.2).

B. Identifying Ca^{2+} Influx Pathways

The simplest method to demonstrate a requirement for Ca^{2+} influx is to show that migration requires the presence of external Ca^{2+} (Strohmeier and Bereiter-Hahn, 1984). Patch clamp recording can then be used to characterize the kinetics, conductance, surface distribution, and pharmacological

properties of the Ca^{2+} channels expressed in the migrating cell (Lee *et al.*, 1999; Maroto *et al.*, 2007). With this knowledge one can then use various treatments to relate particular $[\text{Ca}^{2+}]_i$ changes to specific Ca^{2+} channels activities. One perceived practical limitation of patch clamping is that channel current measurements are restricted to the dorsal surface because it is not possible to patch the ventral “adherent” surface, at least with the traditional patch clamp method (Hamill *et al.*, 1981). In this case, one might argue that because CSK-generated mechanical (traction) forces are transmitted to the substrate purely at ventral surface adhesions, then only mechanosensitive processes in these sites will experience mechanical force and become activated (Mobasheri *et al.*, 2002). However, the traction forces that pull on the substrate via the ventral surface adhesions will also tend to stretch the whole cell for as long as the rear of the cell remains firmly attached to the substrate. Apart from causing the cell to become extended, there are other manifestations of these stretching forces including the smoothing out of membrane folds and microvilli in spreading cells (Erickson and Trinkhaus, 1976), an elastic recoil seen occasionally in some migrating cells as presumably stretching forces exceed adhesive forces (Mandeville and Maxfield, 1997), and even cell rupture/fragmentation that can occur when cell retraction is blocked and the pulling forces exceed the elastic limits of the bilayer (Verkhovsky *et al.*, 1989). Galbraith and Sheetz (1999) have elegantly and directly addressed the issue of force distribution on the ventral and dorsal surfaces by using optical tweezers to measure the membrane tension on the dorsal membrane, and a micromachined device to measure tension generated on the ventral membrane. Their measurements indicate that the dorsal matrix is as effectively linked to the force-generating CSK as the ventral adhesions so tension-sensitive channels located in both the dorsal and ventral surfaces should experience the same stretch. In this case, the MscCa properties measured on the dorsal surface (i.e., their gating kinetics and subsurface distribution) should be important in defining the $[\text{Ca}^{2+}]_i$ dynamics measured during cell migration (Maroto *et al.*, 2007).

C. Ca^{2+} Dependence of *Amoeba* Locomotion

One of the earliest observations implicating Ca^{2+} in amoeboid migration was that lanthanum, a known Ca^{2+} channel inhibitor, blocked movement of *Amoeba discoides* (Hawkes and Hoberton, 1973). Subsequently, microinjection of aequorin (a photoprotein that emits light on Ca^{2+} binding) was used to demonstrate a sustained $[\text{Ca}^{2+}]_i$ elevation in the tail of the amoeba, as well as transient Ca^{2+} influxes in the tips of advancing pseudopods—lowering external $[\text{Ca}^{2+}]_o$ did not immediately reduce rear $[\text{Ca}^{2+}]_i$ but it did block

continued migration (Taylor *et al.*, 1980). This was interpreted as indicating that rear $[Ca^{2+}]_i$ can be maintained by Ca^{2+} release from internal stores, but migration is more sensitive to Ca^{2+} influx into the pseudopod tips (Taylor *et al.*, 1980). In another study, direct injection of fura-2 was used to show that monopodal amoebae developed a continuous $[Ca^{2+}]_i$ gradient increasing from front to rear, whereas polypodal amoebae showed a decrease in $[Ca^{2+}]_i$ in extending pseudopodia, and an increase in retracting pseudopodia (Gollnick *et al.*, 1991; Yumura *et al.*, 1996). Subsequently, intracellular BAPTA, a fast Ca^{2+} buffer, was shown to reduce cell spreading, pseudopodia formation, and amoebae locomotion, and these effects could be reversed by raising $[Ca^{2+}]_o$ (Unterwiesing and Schlatterer, 1995). On the other hand, the same study found that chelation of $[Ca^{2+}]_o$ by the relatively slow Ca^{2+} buffer EGTA did not block pseudopod formation, although it did block the development of any $[Ca^{2+}]_i$ gradient and cell migration. Nebl and Fischer (1997) used recombinant aequorin to demonstrate that chemoattractants induced an increase in $[Ca^{2+}]_i$ that was entirely dependent on Ca^{2+} influx, and speculated that Ca^{2+} -induced actin depolymerization in the rear acted to prevent the formation of stable pseudopod formation in this region of the cell. $[Ca^{2+}]_o$ was shown to be required for shear-flow-induced amoebae motility (but not directionality) and that addition of either EGTA or Gd^{3+} stopped cell movement (Fache *et al.*, 2005). In this case, the effects of external Ca^{2+} were shown to stimulate cell speed by increasing the amplitude, but not the frequency, of both protrusion and retraction events at the cell's leading edge (Fache *et al.*, 2005). Another study based on mutants lacking two major Ca^{2+} -binding proteins in the ER (calreticulum and calnexin) concluded that chemotaxis depended on both Ca^{2+} influx and Ca^{2+} -induced Ca^{2+} release from internal stores (Fisher and Wilczynska, 2006).

Despite the above results, there are also several studies that seem to discount a critical role for Ca^{2+} in amoeboid migration. For example, based on normal chemotaxis seen in a mutant amoeba lacking an IP_3 -like receptor, it was concluded that Ca^{2+} signaling was not required for chemotaxis (Traynor *et al.*, 2000). However, different groups studying the same mutant found that $[Ca^{2+}]_i$ transients dependent on Ca^{2+} influx were not only retained but were required for both chemotaxis and electrotaxis (Schaloske *et al.*, 2005; Shanley *et al.*, 2006). In a different study, it was reported that amoebae can continue their random locomotion with the same speed in the absence of $[Ca^{2+}]_o$ and the presence of 50-mM EGTA or EDTA, apparently ruling out any role for Ca^{2+} influx (Korohoda *et al.*, 2002). However, a more trivial explanation may relate to inadvertent Ca^{2+} leaching from the low profile glass chamber in which both the ventral and dorsal surfaces of the migrating cell make close contact with the glass. Under these conditions, Ca^{2+} may build up in the narrow gaps between the adherent cell and glass

surfaces and reach levels ($\sim 1 \mu\text{M}$) sufficient to support migration (Fisher and Wilczynska, 2006). A similar phenomenon may also account for the apparent lack of external Ca^{2+} dependence of human leukocyte locomotion when they are “chimneying” between closely apposed glass slide and cover slip (Malawista and Boisfleury-Chevance, 1997).

In summary, while most studies indicate that both Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ elevations are required for an amoeba to migrate, the exact role of Ca^{2+} influx in forward protrusion and rear retraction needs to be better defined. There also remains the unresolved issue on whether the reports of amoeba's migration in the absence of $[\text{Ca}^{2+}]_o$ are real or artifactual. In particular, it will be interesting to test whether migration by chimneying is retained in the presence of the faster Ca^{2+} -buffering capacity of BAPTA.

D. Ca^{2+} Dependence of Vertebrate Cell Amoeboid Migration

Newt neutrophils, which are relatively large ($\sim 100 \mu\text{m}$ in diameter) and comparable in size to an amoeba, develop a sustained $[\text{Ca}^{2+}]_i$ gradient that increases from front to rear of the cell as they migrate. Furthermore, spontaneous changes in $[\text{Ca}^{2+}]_i$ gradient direction result in changes in migration direction (Brundage *et al.*, 1991; Gilbert *et al.*, 1994). In contrast, the smaller human neutrophils do not develop a detectable $[\text{Ca}^{2+}]_i$ gradient but instead display $[\text{Ca}^{2+}]_i$ transients when migrating on adhesive substrates (e.g., polylysine, fibronectin, or vitronectin), but not on nonadhesive substrates (Marks and Maxfield, 1990; Hendey and Maxfield, 1993). These $[\text{Ca}^{2+}]_i$ transients can be blocked, along with neutrophil migration, by either removing $[\text{Ca}^{2+}]_o$ or buffering $[\text{Ca}^{2+}]_i$. The $[\text{Ca}^{2+}]_i$ -buffered neutrophils apparently become immobilized because they are unable to retract their rear, which remains anchored to the adhesive substrate. However, they are still capable of spreading, assuming a polarized morphology, and extending their plasma membrane. Furthermore, their motility can be restored by using RGD peptides to block specific integrin attachments to the substrate. Since a similar block of motility could be induced by inhibitors of the Ca^{2+} -dependent phosphatase, calcineurin, it was proposed that this enzyme mediated Ca^{2+} -dependent detachment of the integrin–substrate adhesions (Hendey and Maxfield, 1993). However, the same group later suggested that a more general mechanism for rear detachment may involve Ca^{2+} -increased myosin II contractility (Eddy *et al.*, 2000). A similar Ca^{2+} and RGD sensitivity was seen for neutrophils migrating through a 3D matrigel substrate (Mandeville and Maxfield, 1997), whereas neutrophils migrating on nonadhesive substrates (e.g., glass in the presence of albumin/serum or through cellulose filters) did not display Ca^{2+} transients nor did they require the presence of

external Ca^{2+} or elevations in $[\text{Ca}^{2+}]_i$ in order to migrate (Zigmond *et al.*, 1988; Marks and Maxfield, 1990; Hendey and Maxfield, 1993; Laffafian and Hallet, 1995; Alterafi and Zhelev, 1997). A similar phenomena may occur in the normally gliding fish keratocytes that show an increased frequency of $[\text{Ca}^{2+}]_i$ transients when their rear becomes transiently stuck on the substrate (Lee *et al.*, 1999). An apparently different role for Ca^{2+} signaling involves Ca^{2+} influx-mediated “priming” of nonmotile eosinophils that enables them to undergo transepithelial migration. However, once the cells are primed, they can migrate in the absence of $[\text{Ca}^{2+}]_o$, although they still depend on $[\text{Ca}^{2+}]_i$ elevations (Liu *et al.*, 1999, 2003).

In summary, some of the discrepancies in the Ca^{2+} dependence of neutrophil migration may arise through differences in substrate adhesiveness with the strongest Ca^{2+} dependence seen on sticky substrates, but little or no Ca^{2+} dependence on nonadhesive substrates. At least in this respect, vertebrate cells that display the amoeboid mode may differ from the amoeba, which retains Ca^{2+} dependence even though the amoeba does not depend on integrin adhesion. At least for human neutrophils, $[\text{Ca}^{2+}]_i$ transients rather than gradients appear to be more important in regulating cell migration by promoting rear retraction possibly by increased adhesion disassembly via increases in calcineurin, MLCK, and/or calpain activity.

E. The Role of $[\text{Ca}^{2+}]_i$ Gradients and Transients in Mesenchymal Cell Migration

Cells migrating in the mesenchymal mode can also display sustained $[\text{Ca}^{2+}]_i$ gradients and/or fast transients. Since these different spatio-temporal $[\text{Ca}^{2+}]_i$ dynamics may regulate different steps associated with the mesenchymal migratory cycle, they will be discussed separately below.

1. A Model for Sustained $[\text{Ca}^{2+}]_i$ Gradients

A basic question from the onset is how any cell can maintain a sustained $[\text{Ca}^{2+}]_i$ gradient for as long as several hours in a cytoplasm that allows free diffusion of Ca^{2+} . In particular, the existence of any stable regions of different $[\text{Ca}^{2+}]_i$ within a continuous aqueous medium would seem to disobey the second law of thermodynamics according to which solutes should passively diffuse down their concentration gradient until they reach equilibrium—in the case of Ca^{2+} , this equilibration should occur in seconds or at most minutes. To explain this apparent paradox, Braiman and Priel (2001) proposed that the cell uses energy to actively take up Ca^{2+} uptake into internal stores that can then be passively allowed to leak out into localized regions of the cytoplasm. By this process, combined with a polarized distribution of Ca^{2+} release

channels on a contiguous ER Ca^{2+} store, the cell could create a sustained $[\text{Ca}^{2+}]_i$ elevation in specified subdomains of the cell (Petersen *et al.*, 2001). The interesting aspect of this model is that, one could have uniform Ca^{2+} influx across the cell surface and uniform active uptake by the internal Ca^{2+} stores as long as there was a gradient of Ca^{2+} release from the stores. A further prediction of this model is that if both active uptake and passive leak occur in very close proximity of the membrane, then a subcortical membrane domain of elevated $[\text{Ca}^{2+}]_i$ could be maintained that might go undetected by techniques that only measure global $[\text{Ca}^{2+}]_i$.

2. $[\text{Ca}^{2+}]_i$ Gradients Determine Migrational Directionality

In several cells undergoing mesenchymal migration, $[\text{Ca}^{2+}]_i$ gradients have been shown to be important in determining migration directionality. In particular, Xu *et al.* (2004) observed that migrating cerebellar granule cells develop a $[\text{Ca}^{2+}]_i$ gradient (low front–high back) according to their migration direction. Furthermore, experimental reversal of the $[\text{Ca}^{2+}]_i$ gradient by the application to the front of the cell, an external gradient of various agents that cause $[\text{Ca}^{2+}]_i$ elevation (e.g., chemo-repellant slit2, acetylcholine, and ryanodine) was found to be accompanied by a reversal in migration direction. Similarly, if an external gradient of BAPTA-AM was applied to the back of the cell, again the $[\text{Ca}^{2+}]_i$ gradient and migration direction was reversed. Although some of the same neurons also displayed occasional $[\text{Ca}^{2+}]_i$ transients, no causal relationship was noted between the transients and migration direction (Xu *et al.*, 2004). Similar $[\text{Ca}^{2+}]_i$ gradients related to migration direction have been seen in migrating fibroblasts, kidney epithelial tumor cells, vascular endothelial cells, and prostate tumor cells (Hahn *et al.*, 1992; Schwab *et al.*, 1997; Kimura *et al.*, 2001; Maroto *et al.*, 2007). Moreover, Schwab and colleagues have proposed that the relatively high Ca^{2+} -activated K^+ activity evident in the rear of migrating kidney epithelial tumor cells was a direct consequence of a $[\text{Ca}^{2+}]_i$ gradient rather than polarized surface expression of the K^+ channels (Schwab *et al.*, 1995, 2006). They also proposed that the underlying basis for the $[\text{Ca}^{2+}]_i$ gradient was due to a combination of higher density of Ca^{2+} influx pathways and ER $[\text{Ca}^{2+}]_i$ stores in the cell body compared with the lamellipodia (Schwab *et al.*, 1997). Studies of the highly motile prostate tumor cell line, PC-3, have confirmed some of these ideas (Maroto *et al.*, 2007).

$[\text{Ca}^{2+}]_i$ gradients are seen not only in migrating cells but also in polarized exocrine acinar gland cells where they may regulate unidirectional fluid secretion. In particular, a time-dependent reversal of the $[\text{Ca}^{2+}]_i$ gradient from the luminal to blood side of the acinar cell after acetylcholine (ACh) stimulation has been proposed to be the main basis for a push-pull model for unidirectional fluid secretion (Kasai and Augustine, 1990). In this model,

$[Ca^{2+}]_i$ elevation, first on the luminal cytoplasmic side of the cell causes Cl^- and water efflux into the lumen, then $[Ca^{2+}]_i$ elevation on the blood side of the cell causes Cl^- and water influx from the blood side. Although both cell surfaces express the same Ca^{2+} -activated Cl^- channel, the depolarization that follows ACh stimulation shifts the Cl^- driving force from efflux to influx. A somewhat similar mechanism could presumably underlie the role of ion and water movements in coordinating cell locomotion (Schwab *et al.*, 2006). This possibility seems to be reinforced by the demonstration that aquaporins are selectively expressed in the leading edge of migrating cells (Saadoun *et al.*, 2005). A quite different cell function related to a sustained $[Ca^{2+}]_i$ gradient involves tip growth of fungi in which elevated $[Ca^{2+}]_i$ in the growing tip has been proposed to promote increased insertion of new membrane via exocytosis (Silverman-Gavrila and Lew, 2003). This mechanism would seem unlikely to account for migration directionally since exocytosis predominates at the cell front while endocytosis occurs mainly at the cell rear (Bretscher and Aguado-Velasco, 1998). A more plausible effect of the $[Ca^{2+}]_i$ gradient in promoting cell migration would be to induce polarization of the activities of enzymes regulating actin polymerization/depolymerization, integrin activation/assembly/disassembly, and myosin II contractility (Lauffenburger and Horwitz, 1996; Sheetz *et al.*, 1999; Ridley *et al.*, 2003).

3. $[Ca^{2+}]_i$ Transients

$[Ca^{2+}]_i$ transients have been associated with an even wider variety of other processes including fertilization, cell differentiation, exocytosis, muscle contraction, phagocytosis, and neuronal outgrowth and migration (Berridge *et al.*, 2003). This may be because a $[Ca^{2+}]_i$ transient provides a more efficient and safe way to achieve high levels of $[Ca^{2+}]_i$ compared with steady-state elevations. Furthermore, the temporal component of the signal provides an added dimension in terms of encoding information. $[Ca^{2+}]_i$ transients can take a number of forms in motile cells—they can be highly localized and associated with pseudopod (or bleb) protrusion or retraction, they can spread throughout the cell as a regenerative $[Ca^{2+}]_i$ wave, or they can circumnavigate the perimeter of a cell in a clockwise or anticlockwise direction (Kindzelskii *et al.*, 2004). $[Ca^{2+}]_i$ transients can be generated spontaneously or can be induced experimentally by electrical, chemical, and mechanical stimuli. In particular, it has been shown that direct mechanical stretch of fibroblasts and keratocytes, and osmotic swelling of endothelial cells can induce $[Ca^{2+}]_i$ transients (Arora *et al.*, 1994; Oike *et al.*, 1994; Lee *et al.*, 1999; Wu *et al.*, 1999). $[Ca^{2+}]_i$ transients may also have different initiation sites on different cells and these site may vary within a single cell during the course of the migratory cycle. In particular, the initiation sites of

[Ca²⁺]_i transients have been related to the distribution of membrane rafts and caveolae (i.e., invaginated membrane structures), which contain the molecular signaling machinery required for Ca²⁺ signaling, and can undergo redistribution during migration and specific forms of stimulation. Membrane raft- and caveolae-dependent Ca²⁺ signaling has been observed in cells undergoing both mesenchymal migration (Manes *et al.*, 1999; Isshiki *et al.*, 2002; Parat *et al.*, 2003; Rizzo *et al.*, 2003) and amoeboid migration (Gomez-Mouton *et al.*, 2001; Pierini *et al.*, 2003; Kindzelskii *et al.*, 2004). For example, Isshiki *et al.* (2002) found that the caveolae in quiescent endothelial cells are clustered around the edge of the cell but when stimulated to migrate, either by wounding a cell monolayer or by exposing the cells to laminar shear stress, the caveolae move to the trailing edge of the cell, concomitant with this relocation the sites of Ca²⁺ waves initiation move to the same location (see also Rizzo *et al.*, 2003; Beardsley *et al.*, 2005). In contrast, in human neutrophils lipid rafts and [Ca²⁺]_i transient initiation sites have been localized to the leading edge of the migrating cells, and cholesterol depletion, which disrupts raft structure, was found to block both [Ca²⁺]_i transient initiation and cell migration (Manes *et al.*, 1999; Kindzelskii *et al.*, 2004). Some insight into the different results may be related to the demonstration that both the leading edge and rear of lymphocytes are enriched in lipid components that partition into different raft-like domains (Gomez-Mouton *et al.*, 2001) and that Cav-1, a raft maker, shows a different polarized distribution in endothelial cells depending on whether the cells were migrating on 2D substrate or through a 3D matrix (Parat *et al.*, 2003). In particular, Cave-1 moves from the cell's rear to the cell's front during the switch from the 2D/mesenchymal to the 3D/amoeboid migration modes. These findings are highly intriguing giving that TRPC1, a structural subunit of MscCa (Maroto *et al.*, 2005), colocalizes with Cave-1-associated membrane lipid rafts (Lockwich *et al.*, 2000; Brazier *et al.*, 2003) and has been localized at the leading edge of migrating neutrophils (Kindzelskii *et al.*, 2004) and the rear of migrating prostate tumor cells (Maroto *et al.*, 2007). Together these results indicate that MscCa may redistribute to different regions of the cell surface and perform different, yet critical functions depending on the mode of migration. In this case, MscCa seems to meet the critical criterion of modulating all modes of migration, and unlike integrins, myosin II, calpain, and metalloproteases should not become dispensable following a switch in migration mode.

4. [Ca²⁺]_i Transients Promote Cell Migration but Inhibit Neurite Outgrowth

[Ca²⁺]_i transients have been positively correlated with cell migration in cerebellar granular cells, neutrophils, vascular smooth muscle, keratocytes and astrocytoma cells (Komuro and Rakic, 1996; Lee *et al.*, 1999; Ronde

et al., 2000; Scherberich *et al.*, 2000; Giannone *et al.*, 2002). Furthermore, the cessation of $[Ca^{2+}]_i$ transients has been correlated with the termination of granule cell migration (Kumuda and Komuro, 2004). In contrast, high-frequency $[Ca^{2+}]_i$ transients cause nerve growth cone stalling and axon retraction, while the inhibition of $[Ca^{2+}]_i$ transients stimulates the extension of axonal growth cones and the outgrowth of axonal and dendritic filopodia (Gomez and Spitzer, 1999; Gomez *et al.*, 2001; Robles *et al.*, 2003; Lohmann *et al.*, 2005). The $[Ca^{2+}]_i$ transients in all cases appear to depend on MscCa-mediated Ca^{2+} influx because they are blocked by anti-MscCa agents (Lee *et al.*, 1999; Jacques-Fricke *et al.*, 2006). Furthermore, the opposite effects both appear to depend on calpain activity (Huttenlocher *et al.*, 1997; Robles *et al.*, 2003). However, whereas calpain activity in the cell rear acts to cleave integrin–CSK linkages and in this way promotes rear retraction and cell migration (Huttenlocher *et al.*, 1997), calpain activity in the nerve growth cone and filopodia acts by promoting actin–integrin disengagement at the front of the process, thereby reducing the traction forces required for lamellar protrusion and growth cone translocation (Robles *et al.*, 2003). Interestingly, calpain inhibition in resting neutrophils promotes polarization and random migration whereas it reduces the neutrophil's capacity for directional migration toward chemotactic stimuli (Lokuta *et al.*, 2003). This may occur because constitutive calpain activity in resting neutrophils acts as a negative regulator of polarization and migration, whereas the polarized calpain activity in chemotaxing neutrophils promotes directional persistence in a chemo-attractant gradient.

V. THE ROLE OF MscCa IN CELL MIGRATION

A key issue for all modes of cell migration is the nature of the mechanosensitive molecules that act to coordinate forward cell protrusion with rear cell retraction. An attractive candidate is MscCa that because of its unique ability to transduce membrane stretch/cell extension and transduce this into a Ca^{2+} influx (Guharay and Sachs, 1984; Sachs and Morris, 1998; Hamill and Martinac, 2001; Hamill, 2006) can provide feedback between mechanical forces that tend to extend the cell and the Ca^{2+} -sensitive regulators of force generation and cell–substrate adhesion. The first indirect evidence for a role of MscCa in cell migration was provided by the demonstration that the nonspecific MscCa blocker Gd^{3+} (Yang and Sachs, 1989; Hamill and McBride, 1996) blocked fish keratocyte migration (Lee *et al.*, 1999; Doyle and Lee, 2004; Doyle *et al.*, 2004). Subsequent studies, also using Gd^{3+} , further implicated MscCa in migration of a mouse fibroblast cell line, NIH3T3 (Munevar *et al.*, 2004), and the human fibrosarcoma cell line, HT1080 (Huang *et al.*, 2004).

However, these studies indicated different sites (i.e., front or back) and different actions (i.e., rear retraction, development of traction forces, and disassembly of focal adhesions) for MscCa mediated Ca^{2+} influx, which may partly depend upon different modes of cell migration. Significant limitations in these early studies were the lack of protein identity of MscCa and the absence of MscCa-specific reagents, which have been overcome by the recent identification of the canonical transient receptor potential (TRPC1) (Wes *et al.*, 1995) as an MscCa subunit (Maroto *et al.*, 2005), and the discovery of a highly selective MS channel blocker, GsMTx4 a peptide isolated from the tarantula (*Grammostola spatulata*) venom (Suchyna *et al.*, 2004). Several studies have already implicated TRPC1 in regulating cell migration. For example, Huang *et al.* (2003) showed immunohistologically that TRPC1 was expressed in a punctuate pattern around the cell periphery, and based on Gd^{3+} block proposed that TRPC1 supported $[\text{Ca}^{2+}]_i$ transients and cell migration. Rao *et al.* (2006) while studying an intestinal epithelial cell line demonstrated that suppression of TRPC1 inhibited cell migration, whereas TRPC1 overexpression of TRPC1 enhanced cell migration as measured by an *in vitro* wound closure assay. Maroto *et al.* (2007) characterized MscCa in both motile (PC-3) and nonmotile (LNCaP) human prostate tumor cell lines and found that MscCa displayed the same single-channel conductance, Mg^{2+} and Gd^{3+} sensitivity as the MscCa endogenously expressed in *Xenopus* oocytes identified as formed by TRPC1 (Maroto *et al.*, 2005). Furthermore, MscCa activity was shown to be required for cell migration based on the block by anti-MscCa/TRPC1 agents including GsMTx4, an anti-TRPC1 antibody raised against the external pore region of the channel, siRNA suppression, and overexpression of TRPC1.

Apart from MscCa, there are other Ca^{2+} channels that have been implicated in regulating cell migration including both the T-type (Huang *et al.*, 2004) and L-type voltage-gated Ca^{2+} channels (Yang and Huang, 2005) that may also display mechanosensitivity (Morris and Juranka, Chapter 11, this volume). Also in addition to the TRPCs, which have been implicated in forming MscCa, other TRP subfamily members are expressed in tumor cells and have been implicated in different steps associated with cancer (Peng *et al.*, 2001; Wissenbach *et al.*, 2001; Nilius *et al.*, 2005; Sánchez *et al.*, 2005). Of particular interest is TRPM7 that has been shown to regulate cell adhesion by regulating calpain via Ca^{2+} influx through the channel (Su *et al.*, 2006) and actomyosin contractility via intrinsic kinase activity of TRPM7 (Clark *et al.*, 2006). Although TRPM7 stretch sensitivity has not been directly demonstrated, it has been shown that fluid shear stress-applied human kidney epithelial cells promote membrane trafficking of TRPM7 to the cell surface (Oancea *et al.*, 2006). Given that fluid shear stress can also trigger cell migration (Isshiki *et al.*, 2002), this may provide an additional MS mechanism to regulate cell motility. In this case, it will be interesting to

determine whether the shear-induced increase in TRPM7 surface expression is also dependent on specific integrin engagement (Maroto and Hamill, 2001) and/or related to the flow-induced recruitment of caveolae to specific regions of the migrating cell (Rizzo *et al.*, 2003; Navarro *et al.*, 2004).

There are other classes of gated channels that have been implicated in regulating cell migration including voltage-gated Na^+ (Grimes *et al.*, 1995; Bennett *et al.*, 2004; Onganer and Djamgoz, 2005) and K^+ channels (Laniado *et al.*, 2001) and Ca^{2+} -activated K^+ channels (Schwab *et al.*, 1994). These different channels may participate in a variety of processes to modulate the pattern of cell migration in the same way as different channels act to produce specific patterns of firing and synaptic release in excitable cells. One would expect that MscCa plays a central role in orchestrating the other channels because of its unique ability to transduce internally and externally generated forces into both depolarization and Ca^{2+} influx.

VI. CAN EXTRINSIC MECHANICAL FORCES ACTING ON MscCa SWITCH ON CELL MIGRATION?

A key question is what causes a cell to switch from a nonmotile to a motile phenotype and vice versa? Although there are numerous studies indicating that growth factors including tumor necrosis factor- α and transforming growth factor- β can increase cell motility by promoting the EMT (Bates and Mercurio, 2003; Masszi *et al.*, 2004; Montesano *et al.*, 2005; Nawshad *et al.*, 2005), less well studied is the potential role of extrinsic mechanical forces in turning on cell motility. However, there are at least two key observations that support such a role. In the first place, it has been demonstrated that stationary cell fragments formed from fish keratocytes and lacking a cell nucleus or a microtubular CSK can be stimulated to polarize and undergo persistent locomotion by the application of fluid shear stress or direct mechanical poking (Verkhovsky *et al.*, 1989). Similarly, the application of shear stress to quiescent *Dictyostelium* can cause CSK reorganization and stimulate cell migration (Décavé *et al.*, 2003; Fache *et al.*, 2005). Furthermore, these latter mechanical effects were shown to be critically dependent on the presence of external Ca^{2+} (Fache *et al.*, 2005). One possible explanation is that mechanical forces alter the membrane trafficking (Maroto and Hamill, 2001; Isshiki *et al.*, 2002; Rizzo *et al.*, 2003) and/or the MscCa-gating properties (Hamill and McBride, 1992, 1997; McBride and Hamill, 1992), which in turn alters the $[\text{Ca}^{2+}]_i$ dynamics generated by intrinsic mechanical forces and contributes to further polarization of the cell and directional migration. Several previous studies have already discussed the possible role of the changing mechanical environment in terms of

promoting tumor malignancy, including the possible role of increasing interstitial stress and fluid pressure within a growing tumor (Sarntinoranont *et al.*, 2003) and the increased tumor stiffness due to perturbed vasculature and fibrosis (Paszek *et al.*, 2005) of stimulating increased cell motility and escape from the encapsulated tumor. In this case, MscCa may serve as both a trigger and mediator of tumor progression to malignancy.

Note Added in Proof

Numata, T., Shimizu, T., and Okada, Y. (*Am. J. Physiol.* **292**, C460–C467, 2007) have recently reported that TRPM7 is a stretch- and swelling-activated cation channel expressed in human epithelial cells and is blocked by Gd^{3+} . These results are consistent with the notion that several classes of mechanosensitive channels may regulate different aspects of tumor cell migration (i.e., forward protrusion and rear retraction) depending upon their differential surface distribution and interaction with downstream Ca^{2+} -sensitive effectors.

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Chapter 7

TRPC Family of Ion Channels and Mechanotransduction

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Abstract Here we review recent evidence that indicates members of the canonical transient receptor potential (TRPC) channel family form mechanosensitive (MS) channels. The MS functions of TRPCs may be mechanistically related to their better known functions as store-operated (SOCs) and receptor-operated channels (ROCs). In particular, mechanical forces may be conveyed to TRPC channels through

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“conformational coupling” and/or “Ca²⁺ influx factor” mechanisms that are proposed to transmit information regarding the status of internal Ca²⁺ stores to SOCs located in the plasma membrane. Furthermore, all TRPCs are regulated by receptors coupled to phospholipases (e.g., PLC and PLA₂) that may themselves display mechanosensitivity and modulate channel activity via their generation of lipidic second messengers (e.g., diacylglycerol, lysophospholipids and arachidonic acid). Accordingly, there may be several nonexclusive mechanisms by which mechanical forces may regulate TRPC channels, including direct sensitivity to bilayer deformations (e.g., involving changes in lipid packing, bilayer thickness and/or lateral pressure profile), physical coupling to internal membranes and/or cytoskeletal proteins, and sensitivity to lipidic second messengers generated by MS enzymes. Various strategies that can be used to separate out different MS gating mechanisms and their possible role in each of the TRPCs are discussed.

7.1 Introduction

Mechanotransduction (MT) is a fundamental physiological process by which mechanical forces are transduced into electrical, ionic and/or biochemical signals. MT can span a time scale of milliseconds as in the case of a fast sensory process (e.g., in hearing and touch) to days and even years as in the case of the growth and reorganization of tissues (e.g., skin, muscle and the endothelia) in response to mechanical loading or mechanical stress. Because the plasma membrane forms the interface with the external physical world, it is continually subject to mechanical deformations arising from tissue stretch, compression, gravity, interstitial fluid pressure, fluid shear stress, and also from cytoskeleton (CSK)-generated contractile and tractile forces (Howard et al. 1988; Sachs 1988; Hamill and Martinac 2001; Perbal and Driss-Ecole 2003; Wang and Thampatty 2006; Pickard 2007). Furthermore, the membrane bilayer may be subject to local mechanical distortions caused by the insertion of lipidic second messenger molecules [e.g., diacylglycerol (DAG), arachidonic acid (AA) and lysophospholipids (LPLs)] that act by altering local packing thickness and/or lateral pressure profile, and thereby influence membrane protein conformations with consequences similar to those of global membrane deformations (Martinac et al. 1990; Hamill and Martinac 2001; Perozo et al. 2002; Kung 2005; Martinac 2007; Markin and Sachs 2007; Powl and Lee 2007). It is therefore not surprising to find that a wide range of integral and membrane-associated proteins are specialized to sense and transduce membrane distortions into different homeostatic responses. Here we focus on the seven members of the mammalian canonical transient receptor potential (TRPC1–7) channel family that provide an illustration of how very closely related membrane proteins have evolved different mechanisms for sampling their global and local mechanical environment.

7.2 Distinguishing Direct from Indirect MS Mechanisms

Because TRPCs are gated by a variety of stimuli including direct lipid bilayer stretch as well as by lipidic second messengers that are generated by membrane-associated enzymes that may themselves be mechanosensitive (MS), it is important to establish criteria that may be used to distinguish direct from indirect MS mechanisms of TRPC channel activation. Below we list some tests that may be useful in making this discrimination.

7.2.1 *Stretch Activation of Channels in the Patch*

The most convenient way of demonstrating an MS channel is to use a patch clamp to apply pressure or suction after formation of the giga-seal while simultaneously measuring single channel current activity (Hamill et al. 1981; Guharay and Sachs 1984). The cell-free membrane patch configurations (inside-out and outside-out) can also be used to determine if MS channel activity is retained when the cytoplasmic membrane face is perfused with solutions deficient in soluble second messengers (e.g., Ca^{2+} , cAMP, ATP). However, MS enzymes that generate membrane delimited second messengers may retain their activity following patch excision. Similarly, critical elements of the CSK involved in gating MS channels may be preserved in cell-free membrane patches (Ruknudin et al. 1991). Alternative approaches for testing CSK involvement may involve testing for MS channel activity in membrane patches formed on CSK-deficient membrane blebs induced by ATP depletion or by high ionic strength solution (Zhang et al. 2000; Honoré et al. 2006), and determining how agents that disrupt the CSK elements (e.g., cytochalasins and colchicine) affect the activity of MS channels (Guharay and Sachs 1984; Small and Morris 1994; Honoré et al. 2006).

7.2.2 *Osmotic Swelling and Cell Inflation*

Osmotic stress can also be used to test if a channel is MS either by swelling the cell while recording from a cell-attached patch or from the whole cell (Hamill 1983; Christensen 1987; Sackin 1989; Cemerikic and Sackin 1993; Vanoye and Reuss 1999; Spassova et al. 2006; Numata et al. 2007). The advantage of this approach is that the action of inhibitors and activators can be consistently recognized when assessed on whole cell vs patch currents. However, osmotic cell swelling also activates a number of membrane-associated enzymes, including Src kinase and phospholipase A2 (PLA_2) (Lehtonen and Kinnunen 1995; Cohen 2005a). Furthermore, although some stretch-sensitive channels are sensitive to

osmotic cell swelling and direct cell inflation, others are not (Levina et al. 1999; Vanoye and Reuss 1999; Zhang and Hamill 2000a, 2000b). One basis for this difference is that some cells may possess large excess membrane reserves in the form of folds microvilli and caveola (e.g., *Xenopus* oocytes and skeletal muscle) that can buffer rapid increases in bilayer tension (Zhang and Hamill 2000a, 2000b; Hamill 2006). Conversely, not all channels activated by cell swelling are activated by membrane stretch when applied to the patch (Ackerman et al. 1994; Strotmann et al. 2000).

7.2.3 Gating Kinetics

Another criterion that may be useful in distinguishing direct from indirect mechanisms is the delay time in activation in response to pressure steps applied to the patch with a fast pressure-clamp (McBride and Hamill 1993). Channels that are directly MS should be limited only by the conformational transitions of the channel protein, and may as a consequence show only brief delays (i.e., in the sub-ms or ms range) in their activation and deactivation (McBride and Hamill 1992, 1993). In comparison, channels dependent on enzymatic reactions and/or diffusion of second messenger may be expected to show much longer delays in opening and closing (e.g., ≥ 1 s). These kinetic measurements are best made in the cell-attached or cell-free patch using the pressure clamp to apply pressure steps (1–5 ms rise time) in order to measure the latency in activation and deactivation of the channels (McBride and Hamill 1992, 1993, 1995, 1999; Besch et al. 2002). Rapid activation and deactivation kinetics have been reported for the mechanosensitive Ca^{2+} -permeable cation channel (MscCa) that is formed by TRPCs in *Xenopus* oocytes (Hamill and McBride 1992; McBride and Hamill 1992, 1993) and the expressed TRAAK channel [i.e., a TWIK (tandem of pore domains in a weak inward rectifier K^+ channel)-Related Arachidonic Acid stimulated K $^+$ channel; Honoré et al. 2006]. In contrast, both the MscK expressed in snail neurons, which may also be a two-pore-domain K^+ -channel-like TRAAK (Vandorpe and Morris 1992), and the cation channel formed by TRPC6 show long activation delays of 5–10 s (Small and Morris 1994; Spassova et al. 2006). However, because the delays can be abolished by mechanical and/or chemical CSK disruption it seems more likely that the delays reflect CSK constraint of the bilayer, which prevents rapid transmission of tension to the channel (Small and Morris 1994; Hamill and McBride 1997; Spassova et al. 2006). No studies measuring possible delays in pressure activation of TRPs suspected of being indirectly MS have been performed to date. In the case of the activation of TRPV4, which has been functionally linked to MS PLA_2 generation of AA and its subsequent metabolism to 5', 6'-epoxyeicosatrienoic acid (5'6'-EET) by cytochrome P450 epoxygenase activity (Vriens et al. 2004; Watanabe et al. 2003), whether the apparent lack of stretch sensitivity

was overlooked because of long delays and slow channel activation still needs to be determined (Strotmann et al. 2000).

7.2.4 *The Use of MS Enzyme Inhibitors*

A further strategy for implicating potential MS enzymatic steps in channel activation is to test specific enzyme inhibitor on channel activity. For example, bromoenol lactone (BEL) can be used as a selective blocker of the Ca^{2+} -independent phospholipase A2 (iPLA₂), PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine) is a Src tyrosine kinase blocker, and U73122 can be used to block phospholipase C (PLC) (reviewed by Hamill and Maroto 2007). Using this approach, it has been reported that the stretch sensitivity of a 30 pS cation channel measured in cell-attached patches formed on arterial smooth muscle is abolished by perfusion of the whole muscle cell with U73122, indicating that the channel may derive its stretch sensitivity from a MS iPLA₂ (Park et al. 2003). However, a different study reported that U73122 was ineffective in blocking the stretch sensitivity of a similar 30 pS cation channel measured in inside-out patches isolated from CHO cells transfected with hTRPC6 (Spassova et al. 2006). In another example that involves the excess Ca^{2+} -influx that occurs in dystrophic muscle, and has been proposed to be mediated by TRPC-dependent MS and/or store-operated channels (SOC) (Vandebrouck et al. 2002; Ducret et al. 2006), it was shown that the Ca^{2+} influx could be abolished by BEL (Boittin et al. 2006) and potentiated by the bee venom melittin, a potent activator of PLA₂ (Lindahl et al. 1995; Boittin et al. 2006).

7.2.5 *Reconstitution of MS Channel Activity in Liposomes*

The most unequivocal method for distinguishing direct from indirect mechanosensitivity is to examine whether the detergent-solubilized channel protein retains stretch sensitivity when reconstituted in pure liposomes. So far this criterion has been applied to several MS channels in prokaryotes and MscCa expressed in the frog oocyte (Sukharev et al. 1993, 1994; Sukharev 2002; Kloda and Martinac 2001a, 2001b; Maroto et al. 2005). This approach also offers the potential of definitive evidence on whether lipidic second messengers (e.g., DAG, AA, LPLs and 5'6'-EET) activate the channel by binding directly to the channel protein and/or the surrounding lipid without the requirement of additional proteins and/or enzymatic steps. Furthermore, the same approach may be used to determine whether multi-protein component MS signaling complexes can be functionally reconstituted (e.g., TRPV4/PLA₂/P450 and TRPCs/PLC).

7.3 Extrinsic Regulation of Stretch Sensitivity

It seems highly unlikely that the stretch sensitivity of different membrane channels will be accounted for by a single structural domain analogous to the S-4 voltage sensor domain common to voltage-gated Na^+ , K^+ and Ca^{2+} channels (Hille 2001). This is because even the relatively simple peptide channels gramicidin and alamethicin, which possess dramatically different structures and gating mechanisms, exhibit stretch sensitivity (Opsahl and Webb 1994; Hamill and Martinac 2001; Martinac and Hamill 2002). Furthermore, stretch sensitivity is not a fixed channel property, but rather can undergo significant changes with changing extrinsic conditions. For example, mechanical and/or chemical disruption of the CSK can either enhance or abolish the stretch sensitivity of specific channels (Guharay and Sachs 1984; Hamill and McBride 1992, 1997; Small and Morris 1994; Patel and Honoré 2001; Hamill 2006); changes in bilayer thickness (Martinac and Hamill 2002), membrane voltage (Gu et al. 2001; Morris and Juranka 2007), or dystrophin expression (Franco-Obregon and Lansman 2002) can switch specific MS channels between being stretch-activated to being stretch-inactivated; specific lipids (Patel and Honoré 2001; Chemin et al. 2005), nucleotides (Barsanti et al. 2006a, and references therein) and increased internal acidosis (Honoré et al. 2002; Barsanti et al. 2006b) can convert MS channels into constitutively open 'leak' channels. The basis for many of these changes involves changes in the way the bilayer, CSK and/or extracellular matrix conveys mechanical forces to the channel protein. The practical consequence of this plasticity is that the specific conditions associated with reconstitution and/or heterologous expression may alter the stretch sensitivity of the channel.

7.4 Stretch Sensitivity and Functional MT

Although stretch sensitivity measured in the patch can be used to demonstrate a channel protein is MS at the biophysical level, it cannot prove that the channel functions as a physiological mechanotransducer because conditions associated with the giga-seal formation can increase the stretch sensitivity of the membrane patch (Morris and Horn 1991; Zhang and Hamill 2000b; Hamill 2006). Indeed, many structurally diverse voltage- and receptor-gated channels (e.g., Shaker, L-type Ca^{2+} channels, NMDA-R, S-type K^+ channels), as well as the simple model peptide channels alamethicin and gramicidin A, display stretch sensitivity in patch recordings (Opsahl and Webb 1994; Paoletti and Ascher 1994; Martinac and Hamill 2002; Morris and Juranka 2007). In order to demonstrate functionality one also needs to show that blocking the channel (pharmacologically and/or genetically) inhibits a mechanically induced cellular/physiological process.

7.5 General Properties of TRPCs

The designation TRP originated with the discovery of a *Drosophila* mutant that showed a transient rather than a sustained receptor potential in response to light (Cosens and Manning 1969). This response was subsequently shown to involve a PLC-dependent Ca^{2+} -permeable cation channel (Minke et al. 1975; Montell and Rubin 1989; Minke and Cook 2002). Beginning in the mid 1990s, seven mammalian TRP homologs were identified that, together with the *Drosophila* TRP, make up the canonical TRP (TRPC) subfamily. Other TRP subfamilies include TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPM (melastatin), TRPML (mucolipid), TRPN (NOMPC) and TRPY (yeast); these combine with TRPC to form the TRP superfamily (Montell 2005; Nilius and Voets 2005; Ramsey et al. 2006; Saimi et al. 2007; Nilius et al. 2007). In addition to the TRPCs, specific members of the other TRP subfamilies have also been implicated in MT so that the MS mechanisms discussed here may also apply to these channels (Walker et al. 2000; Palmer et al. 2001; Zhou et al. 2003; Muraki et al. 2003; Nauli and Zhou 2004; O'Neil and Heller 2005; Voets et al. 2005; Saimi et al. 2007; Numata et al. 2007).

The proposed transmembrane topology of TRPCs is reminiscent of voltage-gated channels – sharing six transmembrane spanning helices (TM1–6), cytoplasmic N- and C-termini and a pore region between TM5 and TM6 – but lacking the positively charged residues in the TM4 domain that forms the voltage sensor. TRPC channels also share an invariant sequence in the C-terminal tail called a TRP box (E-W-K-F-A-R), as well as 3–4 N-terminal ankyrin repeats. Although the ankyrin repeats may act as gating springs for MS channels (Howard and Bechstedt 2004) and the positively charged residues in the TRP box may interact directly with the membrane phospholipids, phosphatidylinositol 4,5-bisphosphate (PIP_2) (Rohács et al. 2005) their exact roles remain to be verified (Vazquez et al. 2004a; Owsianik et al. 2006). The TRPCs share very little sequence identity in the region that is C terminal of the TRP box, except for the common feature of calmodulin (CaM)- and inositol 1,4,5-trisphosphate receptor (IP_3R)-binding domains that have been implicated in Ca^{2+} feedback inhibition and activation by store depletion, respectively (Kiselyov et al. 1998; Vaca and Sampieri 2002; Bolotina and Csutora 2005). Based on sequence homology, the TRPCs have been subdivided into the major subgroups of TRPC1/4/5 (showing 65% homology) and TRPC3/6/7 (showing 70–80% homology). TRPC2 is grouped alone because it forms a functional channel in rodents but not in humans (i.e., it is a pseudogene in humans because of the presence of multiple stop codons within its open reading frame).

7.5.1 TRPC Expression

TRPCs are widely expressed in mammalian tissues with some cell types expressing all seven members and others expressing only one or two (Riccio et al. 2002b; Goel et al. 2006; Antoniotti et al. 2006; Hill et al. 2006). Cells that express only one

TRPC may prove particularly useful models for dissecting out specific TRPC functions. However, to justify this role it is necessary to verify that their selective expression is reflected at both the transcriptional and protein levels. This is important because low turnover proteins may require little mRNA, and high mRNA levels need not translate into high membrane protein levels (Andersen and Seilhamer 1997). Another caveat is that TRPC expression patterns can vary significantly during development (see Strübing et al. 2003), and with culture conditions (e.g., presence or absence of growth factors). For example, TRPC1 expression is upregulated by (1) serum deprivation, which leads to increased proliferation of pulmonary arterial smooth muscle cells (Golovina et al. 2001), (2) tumor necrosis factor α , which enhances endothelial cell death (Paria et al. 2003), and (3) vascular injury in vivo, which contributes to human neointimal hyperplasia (Kumar et al. 2006). Also, TRPC6 expression in pulmonary arterial smooth muscle cells is enhanced in idiopathic pulmonary hypertension and by platelet-derived growth factor (Yu et al. 2003, 2004). Compared with mammalian cells there is less information on TRPC expression in lower vertebrates. For example, although a TRPC1 homologue has been identified in *Xenopus* oocytes, a systematic study of expression of other TRPs in lower vertebrates has not yet been carried out (Bobanović et al. 1999).

7.5.2 TRPC Activation and Function: Mechanisms of SOC and ROC

Studies of TRPC activation and function are complicated by their polymodal activation and splice variants that display different activation mechanisms (see Ramsey et al. 2006). However, all TRPCs are regulated by PLC-coupled receptors (i.e., G-protein-coupled receptors or tyrosine kinase receptors). PLC hydrolyzes a component of the bilayer, PIP_2 , into two distinct messengers – the soluble inositol 1,4,5-trisphosphate (InsP_3) that activates the IP_3R in the endoplasmic reticulum (ER) to release Ca^{2+} from internal stores – and the lipophilic DAG, which may regulate TRPs indirectly via protein kinase C (PKC) or by interacting directly with TRPCs in a membrane delimited manner (Hofmann et al. 1999; Delmas et al. 2002; Clapham 2003; Ahmmed et al. 2004; Ramsey et al. 2006). Although all TRPCs could be classified as receptor-operated channels (ROCs, but see Janssen and Kwan 2007), they are more often subdivided into either (1) SOCs, based on their sensitivity to Ca^{2+} store depletion, or (2) ROCs based on both their activation by DAG, AA or their byproducts, and their insensitivity to Ca^{2+} store depletion (Hofmann et al. 1999; Shuttleworth et al. 2004). To be classified as a SOC, the channel should be gated by a variety of procedures that share only the common feature of reducing Ca^{2+} stores, which may or may not depend on IP_3R signaling (see Parekh and Putney 2005). Unfortunately, there have been conflicting reports for all seven TRPCs on whether they function as SOCs, ROCs or both. Furthermore, the nature of the mechanism(s) that activates SOCs remains controversial, with at least two main classes of mechanism in contention. One mechanism depends upon a soluble

Ca^{2+} influx factor (CIF) that is released from depleted Ca^{2+} stores and diffuses to the plasma membrane where it activates the SOC, possibly by releasing inhibitory CaM from iPLA_2 , which generates LPLs and AA (see Bolotina and Csutora 2005). Direct support for this CIF-CaM- iPLA_2 -LPL model has come from the demonstration that functional iPLA_2 is required for SOC activation, displacement of CaM from iPLA_2 activates SOC, and the direct application of LPLs (but not AA) to inside-out patches activates SOCs (Smani et al. 2003, 2004). On the other hand, the generality of this model has been questioned by the finding that BEL, an iPLA_2 inhibitor, does not block thapsigargin-induced Ca^{2+} entry in RBL 2H3 or bone-marrow-derived mast cells (Fensome-Green et al. 2007).

The second main SOC mechanism involves conformational coupling (i.e., “CC” mechanism) between the SOC and a molecule located in the ER that transmits information regarding $[\text{Ca}^{2+}]$ levels in internal stores. This mechanism has received its strongest support with the discovery of STIM (stromal interaction molecule), a resident ER protein with a putative Ca^{2+} binding domain in the ER lumen. Following Ca^{2+} store depletion, STIM has been shown to undergo rapid clustering and increased interactions with elements of the plasma membrane (Liou et al. 2005; Roos et al. 2005). Furthermore, it has been demonstrated that the STIM1 carboxyl-terminus activates native SOC, Ca^{2+} release-activated currents (I_{CRAC}) and TRPC1 channels (Huang et al. 2006). It may turn out that both CIF and CC mechanisms can operate in a redundant manner to activate SOCs, with the CIF mechanism conferring indirect MS on SOC via a MS iPLA_2 (Lehtonen and Kinnunen 1995), and the CC mechanism allowing for direct transmission of mechanical force via a direct STIM–SOC physical connection.

7.5.3 TRPC–TRPC Interactions

Assuming that a cell expresses all seven TRPC subunits, and that four TRPC subunits are required to form a channel (i.e., as a homotetramer or heterotetramer), then there could be as many as 100 different TRPC channels with different neighbor subunit interactions. However, this number would be much lower if only certain TRPC–TRPC combinations are permitted. Two different models have been proposed to underlie the permissible TRPC interactions: the homotypic model, which allows subunits interactions only within each major subgroup – with TRPC1/4/5 combinations forming SOCs and TRPC3/6/7 combinations forming ROCs – (Hofmann et al. 2002; Sinkins et al. 2004); and the heterotypic model, which permits interactions both within and between members of the two subgroups. In a specific heterotypic model developed by Villreal and colleagues it is proposed that TRPC1, 3 and 7 combine to form SOCs (i.e., without participation of TRPC4 and TRPC6) while TRPC3, 4, 6 and 7 combine to form ROCs (i.e., without TRPC1 participation) (Zagranichnaya et al. 2005; Villreal 2006). In this case, the TRPC1 role is limited to forming a SOC and TRPC4 and TRPC6 are limited to forming ROCs. However, in contradiction of an exclusive ROC role for TRPC4, it has been

reported that SOC currents in adrenal cells are abolished by TRPC4 anti-sense treatment (Phillip et al. 2000) and that endothelial cells isolated from TRPC4 knockout mice lack SOC activity (Freichel et al. 2003, 2004). In contrast to the exclusive roles of TRPC1, 4 and 6, TRPC3 and TRPC7 can participate in forming both SOC and ROCs (Zagranichnaya et al. 2005). The validity of the different models has yet to be resolved. However, whereas the homotypic model has been based mostly on gain-of-function results from TRPC overexpression studies, the heterotypic model has been based mostly on loss-of-function results from TRPC suppression studies. At least one complication with the overexpression studies is related to the finding that different levels of specific TRPC expression can influence the function displayed in the transfected cell. In particular, low TRPC3 expression results in increased SOC activity, while high TRPC3 expression results in increased ROC activity (Vazquez et al. 2003). This variation may occur because high expression levels favor TRPC3 homotetrameric channels, whereas low TRPC3 expression allows for heterotetrameric channels with incorporation of endogenous subunits as well as exogenous TRPC (Brereton et al. 2001; Vazquez et al. 2003). Differences in channel function may also arise depending upon whether the cell is permanently or transiently transfected, presumably because stable transfection provides added time for adaptive changes in endogenous protein expression (Lièvremon et al. 2004).

7.5.4 *TRPC Interactions with Scaffolding Proteins*

TRPCs also interact with a variety of regulatory and scaffolding proteins that may add further diversity and segregation of the channels (Ambudkar 2006). In particular, it has been shown that several TRPCs assembly into multi-protein and lipid signaling complexes that result in physical and functional interactions between the plasma membrane, and CSK and ER resident proteins. These interactions may also allow for mechanical forces to be conveyed via a tethered mechanism to gate the channel (Guharay and Sachs 1984; Howard et al. 1988; Hamill and Martinac 2001; Matthews et al. 2007; Cantiello et al. 2007). Alternatively, the interactions may also serve to constrain the development or transmission of bilayer tension to the TRPC channel and thereby “protect” it from being mechanically activated (Small and Morris 1994; Hamill and McBride 1997). For all TRPCs, the C-terminal coiled-coil domains and the N-terminal ankyrin repeats have the potential to mediate protein-CSK interactions. All TRP family members also encode a conserved proline rich sequence LP(P/X)PFN in their C termini that is similar to the consensus binding site for Homer, a scaffold protein that has been shown to facilitate TRPC1 interaction with IP₃R – disruption of which has been proposed to promote SOC activity (Yuan et al. 2003). In particular, TRPC1 mutants lacking Homer protein binding sites show diminished interaction between TRPC1 and IP₃R and the TRPC1 channels are constitutively active. Moreover, co-expression of a dominant-negative form of Homer increases basal TRPC1 channel activity (Yuan et al. 2003). Another protein, I-mfa,

which inhibits helix-loop-helix transcription factors, also binds to TRPC1 and blocks SOC function (Ma et al. 2003). TRPC1 also possesses a dystrophin domain within its C-terminus (Wes et al. 1995) that may allow for interaction with dystrophin – the major CSK protein in skeletal muscle – and this could possibly explain why the absence of dystrophin in Duchenne muscular dystrophic muscle results in TRPC1 channels being abnormally gated open (see Sect. 7.6.1.4). TRPC1 also shows a putative caveolin-1-binding domain that may promote its functional recruitment into lipid rafts and increase SOC activity (Lockwich et al. 2000; Brazier et al. 2003; Ambudkar 2006). As mentioned previously, TRPC1 also interacts with STIM, the putative ER Ca^{2+} sensor molecule that regulates SOC function (Huang et al. 2006). Junctate – another IP_3R -associated protein – interacts with TRPC2, 3 and 5, but apparently not with TRPC1, to regulate their SOC/ROC function (Treves et al. 2004; Stambouliau et al. 2005). In pulmonary endothelial cells, TRPC4 is localized to cell–cell adhesions in cholesterol-rich caveolae and has been shown to interact with the spectrin CSK via the protein 4.1 (Cioffi et al. 2005; Torihashii et al. 2002). Furthermore, either deletion of the putative 4.1 protein binding site on the TRPC4 C-terminus or addition of peptides that competitively bind to that site are able to reduce SOC activity. Another site for TRPC4–CSK interaction involves the PSD-95/disc large protein/zona occludens 1 (PDZ) binding domain located at the TRPC4 distal C-terminus, which binds to the Na^+/H^+ exchange regulatory factor (NHERF) scaffolding protein (Mery et al. 2002; Tang et al. 2000). TRPC6 interacts with the stomatin-like protein podocin, which may modulate its mechano-operated channel (MOC) function in the renal slit diaphragm (Reiser et al. 2005). Interestingly, another stomatin homolog, MEC-2, was proposed to link the putative MS channel to the microtubular CSK in *Caenorhabditis elegans* neurons (Huang et al. 1995) but most recently has been implicated, along with podocin, in regulating MS channel function by forming large protein–cholesterol complexes in the plasma membrane (Huber et al. 2006).

In summary, TRPCs undergo dynamic interactions with various scaffolding proteins that may act to inhibit or promote a particular mode of channel activation. Any one of these interactions may be important in modulating MS of TRPCs by focusing mechanical force on the channel or constraining the channel and/or bilayer from responding to mechanical stretch. It may be that the right combination of TRPC proteins and accessory proteins are needed to produce channels that are not constitutively active but are responsive to factors associated with store depletion and/or mechanical stimulation.

7.5.5 TRPC Single Channel Conductance

Single channel conductance provides the best functional fingerprint of a specific channel, and is superior to identification by whole cell current properties that depend upon multiple factors including single TRPC channel conductance, gating and membrane insertion as well as functional coupling with other channel classes

(i.e., voltage- and Ca^{2+} -activated channels). For example, whole cell currents generated by expression and co-expression of TRPC1/4/5 and/or TRPC3/6/7 subgroup members show I–V relations with dramatically different rectifications (Lintschinger et al. 2000; Strübing et al. 2001). However, these differences may reflect voltage-dependent changes in any one or a combination of the above parameters. Unfortunately, compared with studies of whole cell TRPC generated currents, there have been relatively few studies of the single channel activity that is enhanced by TRPC overexpression or reduced by TRPC suppression. Furthermore, no study to date has distinguished unequivocally between channel currents arising from TRPC homomers or heteromers. To make this distinction one needs to transfect with mutant subunits that produce predictable and measurable changes in channel conductance (or channel block) depending on the subunit stoichiometry within the channel complex (see Hille 2001). Another practical issue for the comparison of different TRPC channel conductance values has been the lack of standardized recording conditions (i.e., pipette solutions with the same composition, and measured over the same voltage range). Nevertheless, a survey of the TRPC single channel values indicates roughly the following order: TRPC3 (65 pS) > TRPC5 (50 pS) > ~ TRPC4 ~ TRPC6 (~30 pS) ≥ TRPC1 (3–20 pS) for estimates made from cell-attached recordings with 100–150 mM Na^+/Cs^+ , 1–4 mM $\text{Ca}^{2+}/\text{Mg}^{2+}$ between –40 and –100 mV (Hofmann et al. 1999; Hurst et al. 1998; Kiselyov et al. 1998; Yamada et al. 2000; Vaca and Sampieri 2002; Liu et al. 2003; Bugaj et al. 2005; Maroto et al. 2005; Inoue et al. 2006; Saleh et al. 2006). The only available estimates for TRPC2 (42 pS) and TRPC7 (60 pS) were made with no divalents (Zufall et al. 2005; Perraud et al. 2001). One basis for the low conductance of TRPC1 compared with TRPC3, 4, 5, 6 and 7 is that TRPC1 lacks the negatively charged aspartate or glutamate residues at analogous positions to D633 in TRPC5 and the other TRPCs, which is situated nine residues from the end of the TM6 domain (Obukhov and Nowycky 2005). Removal of external Ca^{2+} (or Mg^{2+}) has been reported to increase TRPC1 (but not TRPC6) channel conductance and, according to some reports, cause a positive shift in TRPC1 current reversal potential (e.g., Vaca and Sampieri 2002; Maroto et al. 2005; Spassova et al. 2006). The heterogeneity in TRPC1-associated conductance measurements (i.e., 3–20 pS) may also indicate that its conductance is altered when it combines with other subunits. For example, the homomeric TRPC5 channel has a conductance of ~50 pS but the TRPC1/TRPC5 heteromer is reduced to ~10 pS (Strübing et al. 2001). In this case TRPC1 may cause structural distortion of the putative D633 ring formed by the TRPC5 monomeric assembly. The intracellular Mg^{2+} block of TRPC5 at physiological potentials that is relieved at positive potentials also appears to be mediated by D633 (Obukhov and Nowycky 2005). TRPC4 and TRPC6 may have similar voltage-dependent activities because both channels possess aspartate at positions equivalent to D633, and anionic rings at this location may space the properties of TRPC4 and TRPC6. It may also turn out that different TRPCs display multiconductance states some of which are favored by specific conditions. In any case, the conductance values listed above can serve as a baseline for future measurements of the purified/reconstituted TRPCs.

7.5.6 TRPC Pharmacology

Pharmacological tools available to study TRPCs are limited, with different agents reported to block, stimulate or have no effect on different TRPCs (Xu et al. 2005; Ramsey et al. 2006). For example, SKF-96365 blocks TRPC3- and TRPC6-mediated whole cell currents (at $\sim 5 \mu\text{M}$), and is considered a more selective ROC- than SOC-blocker. In contrast, 2-aminoethoxydiphenyl borate (2-APB) blocks TRPC1 ($80 \mu\text{M}$), TRPC5 ($20 \mu\text{M}$) and TRPC6 ($10 \mu\text{M}$) but not TRPC3 ($75 \mu\text{M}$), and is considered a more selective SOC- than ROC-blocker. In the case of Gd^{3+} (and La^{3+}), TRPC1 and TRPC6 are blocked but TRPC4 and TRPC5 are potentiated at $1\text{--}10 \mu\text{M}$ (Jung et al. 2003), while flufenamate blocks TRPC3, TRPC5 and TRPC7 ($100 \mu\text{M}$) but potentiates TRPC6. Amiloride, which is known to block different MscCa, has yet to be tested on TRPC channels (Lane et al. 1991, 1992; Rüscher et al. 1994). The newest anti-MscCa agent, the tarantula venom peptide GsmTX-4 (Suchyna et al. 1998, 2004; Gottlieb et al. 2004; Jacques-Fricke et al. 2006) has more recently been shown to block TRPC channels in mammalian cells but not in *Xenopus* oocytes (Hamill 2006; Spassova et al. 2006). At this stage it would be highly useful to carry out a systematic screen of the various agents reported to target MscCa and/or TRPC, including gentamicin, GsmTX-4, amiloride, 2-APB, amiloride, and SKF-96365 on ROCs as well as SOC (Flemming et al. 2003).

7.6 Evidence of Specific TRPC Mechanosensitivity

There are several lines of evidence indicating specific TRPCs are MS, with the main evidence pointing towards TRPC1, TRPC4 and TRPC6. TRPC1 is generally considered to form a SOC that can be directly activated by LPLs, whereas TRPC4 and TRPC6 appear to form ROCs activated by AA and DAG, respectively. Here we consider whether the same mechanisms underlying SOC and ROC activity and sensitivity to lipidic second messengers is also the basis for their mechanosensitivity.

7.6.1 TRPC1

TRPC1 was the first identified vertebrate TRP homolog (Wes et al. 1995; Zhu et al. 1995); initial heterologous expression of human TRPC1 in CHO and sf9 cells showed enhanced SOC currents (Zitt et al. 1996). However, a subsequent study indicated that hTRPC1 expression in sf9 cells induced a constitutively active nonselective cation channel that was not sensitive to store depletion (Sinkins et al. 1998). This early discrepancy raises the possibility that store sensitivity (and perhaps stretch sensitivity) may depend upon a variety of conditions (e.g., expression levels, presence of

endogenous TRPCs and state of phosphorylation). For example, TRPC1 has multiple serine/threonine phosphorylation sites in the putative pore-forming region and the N- and C-termini, and at least one report indicates that PKC $_{\alpha}$ -dependent phosphorylation of TRPC1 can enhance Ca $^{2+}$ entry induced by store depletion (Ahmmed et al. 2004). Despite the early discrepant reports concerning TRPC1 and SOC function, many studies now point to TRPC1 forming a SOC (Liu et al. 2000, 2003; Xu and Beech 2001; Kunichika et al. 2004; for reviews see Beech 2005; Beech et al. 2003), and in cases where TRPC1 overexpression has not resulted in enhanced SOC (Sinkins et al. 1998; Lintschinger et al. 2000; Strübing et al. 2001) it has been argued that TRPC1 was not trafficked to the membrane (Hofmann et al. 2002). This does not seem to be the case for hTRPC1 when expressed in the oocyte (Brereton et al. 2000). In any case, direct involvement of TRPC1 in forming the highly Ca $^{2+}$ -selective I $_{CRAC}$ seems to be reduced by the recent finding that a novel protein family (i.e., CRAM1 or Orai1) forms I $_{CRAC}$ channels (Peinelt et al. 2006; but see Mori et al. 2002; Huang et al. 2006).

7.6.1.1 A TRPC1 Homologue Expressed in *Xenopus* Oocytes

In 1999, xTRPC1 was cloned from *Xenopus* oocytes and shown to be ~90% identical in sequence to hTRPC1 (Bobanović et al. 1999). An anti-TRPC1 antibody (T1E3) targeted to an extracellular loop of the predicted protein was generated and shown to recognize an 80kDa protein. Immunofluorescent staining indicated an irregular “punctuate” expression pattern of xTRPC1 that was uniformly evident over the animal and vegetal hemispheres. A subsequent patch clamp study also indicated that MscCa was uniformly expressed over both hemispheres (Zhang and Hamill 2000a). This uniform surface expression is in contrast to the polarized expression of the ER and phosphatidylinositol second messenger systems that are more abundant in the animal hemisphere (Callamaras et al. 1998; Jaconi et al. 1999). These results indicate that neither TRPC1 nor MscCa are tightly coupled to ER internal Ca $^{2+}$ stores and IP $_3$ signaling. Originally, it was speculated that the punctuate expression of TRPC1 might reflect discrete channel clusters, but it might also indicate that these channels are localized to the microvilli that make up > 50% of the membrane surface area (Zhang et al. 2000). In another study testing the idea that xTRPC1 forms a SOC, Brereton et al. (2000) found that antisense oligonucleotides targeting different regions of the xTRP1 sequence did not inhibit IP $_3$ -, or thapsigargin-stimulated Ca $^{2+}$ inflow (but see Tomita et al. 1998). Furthermore, overexpression of hTRPC1 did not enhance basal or IP $_3$ -stimulated Ca $^{2+}$ inflow (Brereton et al. 2000). On the other hand, they did see enhancement of a LPA-stimulated Ca $^{2+}$ influx. Interestingly, LPA also enhances a mechanically induced Ca $^{2+}$ influx in a variety of cell types (Ohata et al. 2001). Based on this apparent lack of TRPC1-linked SOC activity, Brereton et al. (2000) proposed that TRPC1 might form the endogenous cation channel activated by the marine toxin, maitotoxin (MTX). However, in another study directly comparing the properties of the endogenous MTX-activated conductance measured in normal liver cells and

the enhanced MTX-activated conductance measured in hTRPC1-transfected liver cells, Brereton et al. (2001) found that the endogenous conductance showed a higher selectivity for Na^+ over Ca^{2+} , and a higher sensitivity to Gd^{3+} block ($K_{50\% \text{ block}} = 1 \mu\text{M}$ vs $3 \mu\text{M}$) compared with the enhanced conductance. These differences may indicate that other endogenous TRPC subunits combine with TRPC1 to form the endogenous MTX-activated conductance, whereas the enhanced MTX-activated conductance is formed exclusively by hTRPC1 homotetramers (Brereton et al. 2001). Finally, unlike in hTRPC1-transfected oocytes, hTRPC1-transfected rat liver cells did show an increased thapsigargin-induced Ca^{2+} inflow (Brereton et al. 2000, 2001).

7.6.1.2 MTX and TRPCs

Evidence from several studies indicates that oocyte MTX-activated conductance may be mediated by MscCa (Bielfeld-Ackermann et al. 1998; Weber et al. 2000; Diakov et al. 2001). In particular, both display the same cation selectivity, both are blocked by amiloride and Gd^{3+} , both are insensitive to flufenamic and niflumic acid, and both have a single channel conductance of $\sim 25 \text{ pS}$ (i.e., when measured in symmetrical 140 mM K^+ and $2 \text{ mM external Ca}^{2+}$). Because MTX is a highly amphipathic molecule (Escobar et al. 1998), it may activate MscCa by changing bilayer mechanics, as has been proposed for other amphipathic agents that activate or modulate MS channel activity (Martinac et al. 1990; Kim 1992; Hamill and McBride 1996; Casado and Ascher 1998; Perozo et al. 2002).

7.6.1.3 TRPC1 and Volume Regulation

To directly test whether TRPC1 might be MS, Chen and Barritt (2003) selectively suppressed TRPC1 expression in rat liver cells and measured the cellular response to osmotic cell swelling. Liver cells are known to express MscCa (Bear 1990), and previous studies had shown that osmotic swelling of epithelial cells activates an MscCa-dependent Ca^{2+} influx that stimulates Ca^{2+} -activated K^+ efflux accompanied by $\text{Cl}^-/\text{H}_2\text{O}$ efflux and regulatory volume decrease (RVD; Christensen 1987). However, contrary to expectations, hypotonic stress actually caused a greater swelling and faster RVD in the TRPC1 suppressed liver cells than in the control liver cells (Chen and Barritt 2003). This may occur because TRPC1 suppression results in a compensatory overexpression of other transport mechanisms that enhance both cell swelling and RVD. It should also be recognized that cell swelling does not always activate MscCa. For example, although hypotonic solution activates a robust Ca^{2+} -independent Cl^- conductance in *Xenopus* oocytes that should contribute to RVD, it fails to activate the endogenous MscCa (Ackerman et al. 1994; Zhang and Hamill 2000a).

7.6.1.4 TRPC1 in Muscular Dystrophy

Both TRPC1 and MscCa are expressed in skeletal muscle and both have been implicated in the muscular degeneration that occurs in Duchenne muscular dystrophy (DMD). In particular, muscle fibers from the *mdx* mouse (i.e., an animal model of DMD) show an increased vulnerability to stretch-induced membrane wounding (Yeung and Allen 2004; Allen et al. 2005) that has been linked to elevated $[Ca^{2+}]_i$ levels caused by increased Ca^{2+} leak channel activity (Fong et al. 1990) and/or abnormal MscCa activity (Franco and Lansman 1990). Based on the observation that the channel activity was increased by thapsigargin-induced store depletion, it was proposed that the channel may also be a SOC belonging to the TRPC family (Vandebrouck et al. 2002, see also Hopf et al. 1996). To test this idea, *mdx* and normal muscle were transfected with anti-sense oligonucleotides designed against the most conserved TRPC regions. The transfected muscles showed a significant reduction in expression of TRPC-1 and -4 but not -6 (all three TRPCs are expressed in normal and *mdx* muscle) and a decrease in Ca^{2+} leak channel activity. Previous studies indicate that MscCa behaves more like a Ca^{2+} leak channel in *mdx* mouse muscle patches (Franco-Obregon and Lansman 2002) and in some *Xenopus* oocyte patches (Reifarth et al. 1999). In a more recent study it has been confirmed that SOC and MscCa in *mdx* mouse muscle display the same single channel conductance and sensitivity to block by Gd^{3+} , SKF96365, 2APB and GsMTx-4 (Ducret et al. 2006). The presence of a dystrophin domain on the C-terminus of TRPC1 (Wes et al. 1995) could explain the shift in MscCa gating mode in *mdx* muscle that lacks dystrophin (Franco-Obregon and Lansman 2002, but see Suchyna and Sachs 2007). However, the findings that TRPC6 and TRPV2 form stretch-sensitive cation channels and are expressed in normal and *mdx* mouse skeletal muscle raises the possibility that several TRPs may contribute to MscCa activity in normal and DMD muscle (Kanzaki et al. 1999; Vandebrouck et al. 2002; Iwata et al. 2003; Muraki et al. 2003; Spassova et al. 2006).

7.6.1.5 TRPC1 Interaction with Polycystins

Further clues pointing to a MS role for TRPC1 relates to the demonstration that TRPC1 interacts with the putative MS channel TRPP2 when they are co-expressed in HEK-293 (Tsiokas et al. 1999; Delmas 2004). TRPP2 is a member of the TRPP family (polycystin) and has been shown to form a Ca^{2+} -permeable cation channel that is mutated in autosomal dominant polycystic kidney disease (ADPKD) (Nauli et al. 2003; Nauli and Zhou 2004; Giamarchi et al. 2006; Cantiello et al. 2007). TRPP2 was originally designated polycystin kidney disease 2 (PKD2) and shown to combine with PKD1, a membrane protein with a large extracellular N-terminal domain that seemed well suited for acting as an extracellular sensing antenna for mechanical forces. Both TRPP2 and PKD1 are localized in the primary cilium of renal epithelial cells that is considered essential for detecting laminar fluid flow (Praetorius and Spring 2005). However, the osmosensitive TRPV4 is also expressed

in renal epithelial cells and may also associate with TRPP2 (Giamarchi et al. 2006). It remains to be determined whether TRPC1 combines with TRPP2 in renal epithelial cells and whether knock-out of TRPC1 and/or TRPV4 blocks fluid flow detection.

7.6.1.6 TRPC1 in Mechanosensory Nerve Endings

If TRPC1 is a mechanosensory channel, it might be expected to be found in specialized mechanosensory nerve endings. To address this issue, Glazebrook et al. (2005) used immunocytochemical techniques to examine the distribution of TRPC1 and TRPC3–7 in the soma, axons and sensory terminals of arterial mechanoreceptors, and found that TRPC1, 3, 4 and 5 (but not TRPC6 and TRPC7) were expressed in the peripheral axons and the mechanosensory terminals. However, only TRPC1 and TRPC3 extended into the low threshold mechanosensory complex endings, with TRPC4 and TRPC5 limited mainly to the major branches of the nerve. Although these results are consistent with TRPC1 (and possibly TRPC3) involvement in baroreception, it was concluded that, because it was not present in all fine terminals, TRPC1 was more likely involved in modulation rather than direct MT. However, it is not clear that all fine endings are capable of transduction. Furthermore, other putative MS proteins (i.e., β and γ ENaC subunits) are expressed in baroreceptor nerve terminals (Drummond et al. 1998), in which case different classes of MS channels (i.e., ENaC and TRPC) may mediate MT in different mechanosensory nerves.

7.6.1.7 TRPC1 Involvement in Wound Closure and Cell Migration

For a cell to migrate there must be coordination between the mechanical forces that propel the cell forward and the mechanisms that promote retraction of the cell rear. The first study to implicate TRPC1 in cell migration was by Moore et al. (1998). They proposed that shape changes induced in endothelial cells by activation of TRPC1 were a necessary step for angiogenesis and cell migration. In another study, it was demonstrated that TRPC1 overexpression promoted, while TRPC1 suppression inhibited, intestinal cell migration as measured by wound closure assay (Rao et al. 2006). Based on the proposal that MscCa regulates fish keratocyte cell migration (Lee et al. 1999), and identification of TRPC1 as an MscCa subunit (Maroto et al. 2005), the role of TRPC1 in migration of the highly invasive/metastatic prostate tumor cell line PC-3 has been tested. TRPC1 activity was shown to be essential for PC-3 cell migration and, in particular, Gd^{3+} , GsMTx-4, anti-TRPC1 antibody and siRNA targeting of TRPC1 were shown to block PC-3 migration by inhibiting the Ca^{2+} dynamics that coordinated cell migration (R. Maroto et al., manuscript submitted). However, again TRPC1 may not be the only TRP channel involved in this function since TRPC6 and TRPM7 have recently been reported to be stretch-activated channels (Spasova et al. 2006; Numata et al. 2007). Irrespective of the

exact molecular identity of MscCa, it seems that this channel may be a more promising target for blocking tumor cell invasion and metastasis than integrins and metalloproteinases. This is because when a tumor cell switches from mesenchymal to amoeboid migration mode it appears to remain dependent upon Ca^{2+} influx via MscCa, whereas it becomes relatively independent of integrin and metalloproteinase activity (for review, see Maroto and Hamill 2007).

7.6.1.8 Reconstitution of xTRPC1 in Liposomes

Perhaps the most direct evidence for an MS role for TRPC1 comes from studies in which the proteins forming the oocyte MscCa were detergent-solubilized, fractionated by FPLC, reconstituted in liposomes and assayed for MscCa activity using patch recording (Maroto et al. (2005). A specific protein fraction that ran with a conductivity of 16 mS cm^{-1} was shown to reconstitute the highest MscCa activity, and silver-stained gels indicated a highly abundant 80 kDa protein. Based on previous studies that identified xTRPC1 and hTRPC1 as forming an ~80 kDa protein when expressed in oocytes (Bobanović et al. 1999; Brereton et al. 2000), immunological methods were used to demonstrate that TRPC1 was present in the MscCa active fraction. Furthermore, heterologous expression of hTRPC was shown to increase the MscCa activity expressed in the transfected oocyte, whereas TRPC1-antisense reduced endogenous MscCa activity (Maroto et al. 2005). Despite the almost tenfold increase in current density in the TRPC1-injected oocyte, the channel activation and deactivation kinetics in the two patches were similar, at least in some patches. On the other hand, in some cases the kinetics of the TRPC1-dependent channels show delayed activation and deactivation kinetics (Hamill and Maroto 2007). The basis for this heterogeneity in kinetics of TRPC1 channels remains unclear but may reflect local differences in the underlying CSK and/or bilayer or even the MscCa subunit composition that occurs with TRPC1 overexpression. Maroto et al. (2005) also demonstrated that hTRPC1 expression in CHO cells results in increased MscCa activity, consistent with a ~fivefold greater increase in channel density. Furthermore, the presence of endogenous MscCa activity is consistent with previous reports that indicate CHO cells express TRPC1 along with TRPC2, 3, 4, 5 and 6 (Vaca and Sampieri 2002).

7.6.2 TRPC2

So far there have been no studies addressing the possibility that TRPC2 is an MS channel. However, evidence does indicate that TRPC2 may function either as a ROC or a SOC depending upon cell type (Vannier et al. 1999; Gailly and Colson-Van Schoor 2001; Chu et al. 2004; Zufall et al. 2005). For example, because TRPC2^{-/-} mice fail to display gender discrimination, the channel has

been implicated in pheromone detection in the rodent vomeronasal organ (VNO) (Liman et al. 1999; Zufall et al. 2005). Furthermore, because a DAG-activated channel in VNO neurons is down-regulated in TRPC2^{-/-} mice and TRPC2 is localized in sensory microvilli that lack Ca²⁺ stores, it would seem that TRPC2 functions as a ROC rather than a SOC, at least in VNO neurons (Spehr et al 2002; Zufall et al. 2005). On the other hand, in erythroblasts, and possibly sperm, TRPC2 has been reported to be activated by store depletion. In both cell types, long splice variants of TRPC2 were detected (Yildrin et al. 2003), whereas VNO neurons express a short splice variant (Chu et al. 2002; Hofmann et al. 2000). In hematopoiesis, erythropoietin is proposed to modulate Ca²⁺ influx via TRPC2 in possible combination with TRPC6 (Chu et al. 2002, 2004). In sperm, TRPC2 may participate in the acrosome reaction based on its inhibition by a TRPC2 antibody (Jungnickel et al. 2001). However, the fact that TRPC2^{-/-} mice display normal fertility raises serious doubts regarding this role (Stambouliau et al. 2005).

7.6.3 TRPC3

TRPC3 co-localizes with TRPC1 in specialized mechanosensory nerve endings, indicating that these two TRPCs may combine to form an MS channel (see Sect. 7.6.1.6). Because TRPC3 is activated by the DAG analog 1-oleoyl-2-acetyl glycerol (OAG) in a direct manner like TRPC6 (Hofmann et al. 1999), it would seem likely that it may also be sensitive to direct membrane stretch like TRPC6 (Spasova et al. 2006). However, TRPC3, unlike TRPC6, can also contribute to forming SOCs (Zitt et al. 1997; Hofmann et al. 1999; Kamouchi et al. 1999; Trebak et al. 2002; Vasquez et al. 2001; Liu et al. 2005; Groschner and Rosker 2005; Zagranichnaya et al. 2005; Kawasaki et al. 2006), and whether TRPC3 forms a SOC or a ROC has been shown to depend on levels of TRPC3 expression, indicating that subunit stoichiometry may determine activation mode (Vasquez et al. 2003; Putney et al. 2004). Finally, suppression of TRPC3 in cerebral arterial smooth muscle, while suppressing pyridine receptor-induced depolarization, does not appear to alter pressure increased depolarization and contraction, which therefore might be dependent on TRPC6 alone (Reading et al. 2005).

7.6.4 TRPC4

There is disagreement on whether TRPC4 functions as a SOC and/or ROC (Philipp et al. 1998; Tomita et al. 1998; McKay et al. 2000; Plant and Shaefer 2005). However, at least two studies by the Villreal group indicate that TRPC4 forms a ROC activated by AA rather than by DAG as in the case of TRPC3/6/7 and TRPC2 (Wu et al. 2002; Zagranichnaya et al. 2005). In particular, using siRNA and antisense strategies to reduce endogenous TRPC4 expression, TRPC4 was shown to be

required for AA-induced Ca^{2+} oscillations but not for SOC function. This AA activation may have implications for the mechanosensitivity of TRPC4 since AA has been shown to activate/modulate a variety of MS channels by directly altering the mechanical properties of the bilayer surrounding the channel (Kim 1992; Hamill and McBride 1996; Casado and Ascher 1998; Patel and Honoré 2001). Since AA is produced by PLA_2 , which is itself MS (Lehtonen and Kinnunen 1995), TRPC4 may derive its mechanosensitivity from this enzyme in addition to possibly being directly sensitive to bilayer stretch. Studies of TRPC4^{-/-} mice indicate that TRPC4 is an essential determinant of endothelial vascular tone and endothelial permeability as well neurotransmitter release from central neurons (reviewed by Freichel et al. 2004).

7.6.5 TRPC5

The human TRPC5 encodes a protein that is very similar to TRPC4 in its first ~700 amino acids but shows more variability in final C-terminal ~200 amino acids (Sossey-Alaoui et al. 1999; Zeng et al. 2004). Both TRPC5 and TRPC4 differ from other TRPCs in terms of possessing a C-terminal VTTRL motif that binds to PDZ domains of the scaffolding proteins EBP50 (NHERF1). However, co-expression and deletion experiments have shown that the VTTRL motif is not necessary for TRPC5 activation although it may mediate the EBP50 modulatory effects on TRPC5 activation kinetics (Obukhov and Nowycky 2004). TRPC5 (and 4) differ from the other TRPCs in that La^{3+} and Gd^{3+} cause potentiation at micromolar concentrations and block only at higher concentrations (Schaefer et al. 2000; Strübing et al. 2001; Jung et al. 2003). On this basis alone, TRPC5 and TRPC4 homotetramers would seem to be excluded from forming MscCa because Gd^{3+} has usually been reported to block MscCa at 1–10 μM (Yang and Sachs 1989; Hamill and McBride 1996). 2-APB blocks TRPC5 as well as the activating effect of Gd^{3+} possibly by directly occluding the Gd^{3+} activation site (Xu et al. 2005). TRPC5 (and TRPC4) also differ from TRPC3/6/7 in that they are not activated directly by DAG (Hofmann et al. 1999; Schaefer et al. 2000; Venkatachalam et al. 2003). However, TRPC5 is activated by LPLs including LPC when applied to excised membrane patches, but not by the fatty acid AA (Flemming et al. 2006; Beech 2006). This latter result would seem to contradict the idea that TRPC4 forms the AA-activated ROC, ARC, unless the two closely related TRPCs differ significantly in their AA sensitivity (Zagranichnaya et al. 2005).

The most intriguing functional evidence implicating TRPC5 as a putative MscCa comes from the demonstration that TRPC5, like MscCa, functions as negative regulator of neurite outgrowth (Calabrese et al. 1999; Greka et al. 2003; Hui et al. 2006; Jacques-Fricke et al. 2006; Pellegrino and Pelligrini 2007). In particular, MscCa blockers, including gentamicin, Gd^{3+} and GsmTX-4, potentiate neurite outgrowth (Calabrese et al. 1999; Jacques-Fricke et al. 2006) as does expression of a TRPC5 dominant-negative pore mutant. In contrast, overexpression of TRPC5

suppresses neurite outgrowth (Greka et al. 2003; Hui et al. 2006). Although it is tempting to suggest that TRPC5 may form MscCa in neurites, the stretch sensitivity of TRPC5 and its sensitivity to block by GsmTX-4 needs to be directly tested. Furthermore, because neurite outgrowth is potentiated by ruthenium red (a TRPV4 blocker) and suppressed by the specific TRPV4 agonist 4 α -phorbol 12, 12-didecanoate, it has been suggested that TRPV4 forms the MscCa (Jacques-Fricke et al. 2006). Furthermore, in contrast to its proposed role in suppressing cell motility, TRPC5, possibly in combination with TRPC1, has also been implicated in mediating sphingosine 1-phosphate-stimulated smooth muscle cell migration (Xu et al. 2006).

7.6.6 TRPC6

The general consensus is that TRPC6 forms a ROC that is directly activated by DAG, and is insensitive to activation by IP₃ and Ca²⁺ store depletion (Boulay et al. 1997; Hofmann 1999; Estacion et al. 2004; Zagranichnaya et al. 2005; Zhang et al. 2006). Although TRPC6 is a member of the TRPC3/6/7 subfamily it shows distinct functional and structural properties. Functionally, while TRPC6 forms only a ROC, TRPC3 and TRPC7 appear capable of participating in forming both ROCs and SOCs (Zagranichnaya et al. 2005). Structurally, whereas TRPC6 carries two extracellular glycosylation sites, TRPC3 carries only one (Dietrich et al. 2003). Furthermore, exogenously expressed TRPC6 shows low basal activity compared with TRPC3, and elimination of the extra glycosylation site that is missing in TRPC3, transforms TRPC6 into a constitutively active TRPC-3 like channel. Conversely, engineering of an additional glycosylation site in TRPC3 markedly reduces TRPC3 basal activity. It will be interesting to determine how these manipulations alter the apparent MS functions of TRPC6 described below.

7.6.6.1 TRPC6 Role in Myogenic Tone

TRPC6 is proposed to mediate the depolarization and constriction of small arteries and arterioles in response to adrenergic stimulation (Inoue et al. 2001, 2006; Jung et al. 2002) and elevation of intravascular pressure consistent with TRPC6 forming a MOC as well as a ROC (Welsh et al. 2000, 2002). The cationic current activated by pressure in vascular smooth muscle is suppressed by antisense-DNA to TRPC6 (Welsh et al. 2000). Furthermore, because cation entry was stimulated by OAG and inhibited by PLC inhibitor (Park et al. 2003), it was proposed that TRPC6 forms a MS channel that is activated indirectly by pressure according to the pathway:

\uparrow Intravascular pressure \rightarrow \uparrow PLC \rightarrow \uparrow [DAG] \rightarrow \uparrow TRPC \rightarrow \uparrow [Ca²⁺] \rightarrow \uparrow myogenic tone.

In this scheme it is PLC rather than TRPC that is MS and, since all TRPCs are coupled to PLC-dependent receptors, this would imply that all TRPC could display some degree of mechanosensitivity. However, while there are reports that PLC can be mechanically stimulated independent of external Ca^{2+} (Rosales et al. 1997; Mitchell et al. 1997; Moore et al. 2002), there are more cases that indicate that the mechanosensitivity of PLC derives from stimulation by Ca^{2+} influx via MscCa (Matsumoto et al. 1995; Ryan et al. 2000; Ruwhof et al. 2001). In this case, it becomes important to demonstrate that TRPC6 can be mechanically activated in the absence of external Ca^{2+} (e.g., using Ba^{2+}). There is other evidence to indicate that TRPC6 may be coupled to other MS enzymes. For example, TRPC6 is similar to TRPV4 in that it is activated by 20-hydroxyeicosatetraenoic acid (20-HETE), which is the dominate AA metabolite produced by cytochrome P-450 w-hydroxylase enzymes (Basora et al. 2003). TRPC6 may also be activated by Src family protein tyrosine kinase-mediated tyrosine phosphorylation (Welsh et al. 2002). Indeed, PP2 a specific inhibitor of Src PTKs, abolishes TRPC6 (and TRPC3) activation and strongly inhibits OAG-induced Ca^{2+} entry (Soboloff et al. 2005). OAG may operate solely through TRPC6 homomers, whereas vasopressin may act on OAG-insensitive TRPC heteromers (e.g., formed by TRPC1 and TRPC6). At least consistent with this last possibility is evidence of co-immunoprecipitation between TRPC1 and TRPC6 (Soboloff et al. 2005). A further complication is that DAG-dependent activation of PKC appears to stimulate the myogenic channels based on their block by the PKC inhibitor chelerythrine (Sligh et al. 2002), whereas PKC activation seems to inhibit TRPC6 channels, which would seem more consistent with direct activation by DAG/OAG (Soboloff et al. 2005).

Despite the above evidence implicating TRPC6 as the “myogenic” channel, TRPC6-deficient mice show enhanced rather than reduced myotonic tone and increased rather than reduced responsiveness to constrictor agonist in small arteries. These effects result in both a higher elevated mean arterial blood pressure and a shift in the onset of the myogenic tone towards lower intravascular pressures, again opposite to what would be expected if TRPC6 were critical for myoconstriction (Dietrich et al. 2005). Furthermore, isolated smooth muscle from TRPC6^{-/-} mice shows increased basal cation entry and more depolarized resting potentials, but both effects are blocked if the muscles are also transfected with siRNA targeting TRPC3. Based on this latter observation, it was suggested that constitutively active TRPC3 channels are upregulated in TRPC6^{-/-} mice. However, the TRPC3 subunits are unable to functionally replace the lost TRPC6 function that involves suppression of high basal TRPC3 activity (i.e., the TRPC3/6 heteromer is a more tightly regulated ROC and/or MOC). In summary, although evidence indicates that TRPC6 may be a pressure- or stretch-sensitive channel and contribute to MOC, the TRPC6 knockout mouse indicates a phenotype that cannot be explained if TRPC6 alone forms the vasoconstrictor channel. It may also be relevant that another study could find no evidence that Gd^{3+} -sensitive MscCa contributes to myogenic tone in isolated arterioles from rat skeletal muscle (Bakker et al. 1999).

In the most direct study concerning TRPC6 mechanosensitivity, a stretch-activated channel current with a conductance of 25 pS (measured at +60 mV)

was activated in cell-attached patches formed on HEK293 cells transfected with hTRPC6 with a significant delay (~5 s) in turn on and turn off following a brief (2 s) pressure pulse (Spasova et al (2006). Although these long delays could indicate an indirect mechanism of stretch activation, possibly involving MS PLC (see Sect. 7.2.3), it was found that treatment of cells with cytochalasin D reduced the delays and increased stretch sensitivity, which is more consistent with the actin CSK acting as a mechanical constraint that acts to delay the transmission of tension to the bilayer. It was also found that either hypoosmotic cell swelling or application of OAG to TRPC6-transfected cells activated whole cell cation conductance that was not blocked by the PLC inhibitor U73122, apparently ruling out an indirect mechanism involving MS PLC as was previously implied (Park et al. 2003).

7.6.6.2 TRPC6 Role in Kidney Disease

Autosomal dominant focal segmental glomerulosclerosis (FSGS) is a kidney disease that leads to progressive renal kidney failure characterized by leakage of plasma proteins like albumin into the urine (proteinuria). Recently, mutations in TRPC6 were associated with familial FSGS and implicated in aberrant calcium signaling that leads to podocyte injury (Winn et al. 2005; Reiser et al. 2005). Furthermore, two of the mutants were demonstrated to be gain-of-function mutations that produce larger ROCs than the ROC currents measured in wild type TRPC6-expressing HEK-293 cells. Ultra-filtration of plasma by the renal glomeruli is mediated mainly by the podocyte, which is an epithelial cell that lies external to the glomerular basement membrane (GBM) and lines the outer endothelium of the capillary tuft located inside the Bowman's capsule. The podocyte covers the GBM and forms interdigitating foot processes that are connected by slit diaphragms, which are ultra-thin membrane structures that form a zipper-like structure at the center of the slit with pores smaller than albumin (Tryggvason and Wartovaara 2004; Kriz 2005). The podocyte-specific proteins nephrin and podocin are localized in the slit diaphragm, and the extracellular domains of nephrin molecules of neighboring foot processes interact to form the zipper structure. Podocin, a member of the stomatin family, is a scaffolding protein that accumulates in lipid rafts and interacts with the cytoplasmic domain of nephrin (Durvasula and Shankland 2006). Both nephrin and podocin have been shown to be mutated in different familial forms of FSGD. Furthermore, TRPC6 interacts with both nephrin and podocin, and a nephrin-deficiency in mice leads to overexpression and mislocalization of TRPC6 in podocytes as well as disruption of the slit diaphragm (Reiser et al. 2005). Mechanical forces play an important role in ultra-filtration, both in terms of the high transmural distending forces arising from the capillary perfusion pressure, as well as the intrinsic forces generated by the contractile actin network in the foot process that control, in a Ca-dependent manner, the width of the filtration slits. As a consequence, TRPC6 may act as the central signaling component mediating pressure-induced constriction of the slit.

In summary, two quite diverse physiological functions, myogenic tone and renal ultrafiltration, implicate TRPC6 as an MS channel, and recent evidence indicates that TRPC6 may be directly activated by stretch applied to the patch.

7.6.7 TRPC7

Since TRPC7 belongs to the same subfamily as TRPC6, and also forms a ROC activated by DAG/OAG, it might be expected to display the same direct stretch sensitivity to Ca^{2+} block as reported for TRPC6. Immunoprecipitation and electrophysiological experiments indicate that TRPC6 and TRPC7 can co-assemble to form channel complexes in A7r5 vascular smooth muscle cells (Maruyama et al. 2006). However, the same study also demonstrated that the co-assembly of TRPC7 (or TRPC73) with TRPC6 can change specific channel properties compared with the homomeric TRPC6 channel. For example, whereas increasing external Ca^{2+} from 0.05 to 1 mM suppresses currents in HEK cells transfected with TRPC7 (or TRPC3) alone, or with TRPC6/7 (or TRPC3/6) in combination, it fails to suppress currents in TRPC6-transfected cells. Therefore, apart from the constitutive opening seen with TRPC3 but not TRPC6 (see Sect. 7.5.3), TRPC3/6/7 subfamily members differ in their sensitivity Ca^{2+} block. Other studies indicate even more profound differences between TRPC7 and TRPC6 functions. For example, based on overexpression in HEK cells, it was concluded that mouse TRPC7 forms a ROC, whereas human TRPC7 forms a SOC (Okada et al. 1999; Riccio et al. 2002a). In this case, the initial explanation was that a proline at position 111 in mTRPC7 was replaced by leucine in the hTRPC7. However, hTRPC7 suppression/knockout experiments indicate that TRPC7 is required for both the endogenous SOC and ROC in HEK293 cells (Lièvremonet et al. 2004; Zagranichnaya et al. 2005). Furthermore, when hTRPC7 (with leucine at position 111) was transiently expressed in HEK293 cells it enhanced ROC, but when it was stably expressed it enhanced both ROC and SOC (Lièvremonet et al. 2004). In this case, the explanation was that stable transfection allowed for a time-dependent up-regulation of other ancillary components that were required to couple TRPC7 to store depletion (Lièvremonet et al. 2004). On the other hand, although hTRPC7 suppression in DT40 B-cells also reduced receptor/DAG-activated and store-operated Ca^{2+} entry, the latter effect appeared to arise because of increased Ca^{2+} stores and the greater difficulty in depleting them to activate SOC (Lièvremonet et al. 2005). Indeed, when Ca^{2+} stores were more effectively depleted (i.e., with a combination of IP_3 and calcium chelator) there was no difference in SOC activation between wild type and TRPC7^{-/-} cells (Lièvremonet et al. 2005). Similar findings have been reported for TRPC7 suppression in human keratinocytes (Beck et al. 2006). A still further complication is that, in cells lacking the IP_3R , the OAG-activated current is absent but can be restored by transient IP_3R expression or by overexpression of TRPC7 (Vazquez et al. 2006). This was taken to indicate that the endogenous TRPC7 needs to interact with endogenous

proteins including regulatory IP_3R but when TRPC7 is overexpressed the other proteins are not required for OAG activation.

The above review of the TRPC literature indicates the importance of measuring directly the stretch sensitivity of different TRP channels under conditions in which the stoichiometry and molecular nature of the TRPCs forming the channel complex are well defined.

7.7 Conclusions

At least three basic mechanisms, referred to as “bilayer”, “conformational coupling” and “enzymatic”, may confer mechanosensitivity on TRPCs. The bilayer mechanism should operate if the TRPC channel, in shifting between closed and open states, undergoes a change in its membrane occupied area, thickness and/or cross-sectional shape. Any one of these changes would confer mechanosensitivity on the channel. A bilayer mechanism may also underlie the ability of lipidic second messengers (e.g., DAG/OAG, LPL, AA and 5'-EET) to directly activate TRPC channels by inserting in the bilayer to alter its local bilayer packing, curvature and/or the lateral pressure profile. The only unequivocal way to demonstrate that a bilayer mechanism operates is to show that stretch sensitivity is retained when the purified channel protein is reconstituted in liposomes. After this stage, one can go on to measure channel activity as a function of changing bilayer thickness (i.e., by using phospholipids with different acyl length chains) and local curvature/pressure profile (i.e., by using lysophospholipids with different shapes) (Perozo et al. 2002).

The second mechanism involves conformational coupling (CC), which has been evoked to account for TRPC sensitivity to depletion of internal Ca^{2+} stores. CC was originally used to explain excitation–contraction (E–C) coupling involving the physical coupling between L-type Ca^{2+} channels (i.e., dihydropyridine receptors, DHPR) in the plasma membrane and ryanodine receptors (RyR1) that release Ca^{2+} from the sarcoplasmic reticulum (SR) (Protasi 2002). Subsequently, a retrograde form of CC was discovered between the same two proteins that regulate the organization of the DHPR into tetrads and the magnitude of the Ca^{2+} current carried by DHPR (Wang et al. 2001; Paolini et al. 2004; Yin et al. 2005). Another form of CC was demonstrated associated with physiological stimuli that do not deplete Ca^{2+} stores yet activate Ca^{2+} entry through channels referred to as excitation-coupled Ca^{2+} entry channels to distinguish them from SOC (Cherednichenko et al. 2004). Interestingly, RyR1 is functionally coupled to both TRPC1-dependent SOC and TRPC3-dependent SR Ca^{2+} release (Sampieri et al. 2005; Lee et al. 2006).

A key issue for all forms of CC is whether the direct physical link that conveys mechanical conformational energy from one protein to another can also act as a pathway to either focus applied mechanical forces on the channel or alternatively constrain the channel from responding to mechanical forces generated within the bilayer. Another possibility is that reorganization or clustering of the resident ER

protein (i.e., STIM) that senses Ca^{2+} stores may alter channel mechanosensitivity by increasing the strength of CC (Kwan et al. 2003).

Some insights into these possibilities can be provided by the process of “membrane blebbing”, which involves decoupling of the plasma membrane from the underlying CSK, and has been shown to either increase or decrease the mechanosensitivity of MS channels depending upon the channel (Hamill and McBride 1997; Hamill 2006). Since membrane blebbing would also be expected to disrupt any dynamic interactions between TRPC channels and scaffolding proteins it should alter TRPC function. In one case it has been reported that Ca^{2+} store depletion after, but not before, formation of a tight seal is effective in blocking the activation of SOC channels in frog oocyte patches (Yao et al. 1999). Presumably, this occurs because the sealing process physically decouples the channels from ER proteins that sense internal Ca^{2+} stores. Tight seal formation using strong suction can also reduce MscCa mechanosensitivity and gating kinetics, possibly by a related mechanism (Hamill and McBride 1992). On the other hand, it has been reported that I_{CRAC} is retained following cell “ballooning” (i.e., a form of reversible membrane blebbing) indicating that the coupling between the channel and the Ca^{2+} sensor STIM may be relatively resistant to decoupling (Bakowski et al. 2001). In any case, in order to directly demonstrate a role for CC in mechanosensitivity, one needs to show that stretch sensitivity can be altered in mutants in which TRPC–ancillary protein interactions are disrupted (see Sect. 7.5.4).

The third mechanism of mechanosensitivity relates to functional coupling between TRPCs and putative MS enzymes. Evidence indicates that the PLA_2 and Src kinase may be MS, and both enzymes have been implicated in conferring mechanosensitivity on TRPV4 (Xu et al. 2003; Vriens et al. 2004; Cohen 2005a, 2005b). PLA_2 and Src kinase have also been implicated in the activation of TRPC-mediated SOC and ROC activities (Hisatsune et al. 2004; Bolotina and Csutora 2005; Vazquez et al. 2004b). There is also evidence that indicates PLC may be MS (Brophy et al. 1993), with some reports indicating that the mechanosensitivity depends upon Ca^{2+} influx (Basavappa et al. 1988; Matsumoto et al. 1995; Ryan et al. 2000; Ruwhof et al. 2001; Alexander et al. 2004) and others indicating independence of external Ca^{2+} and Ca^{2+} influx (Mitchell et al. 1997; Rosales et al. 1997; Moore et al. 2002). In either case, the combined evidence indicates that mechanical forces transduced by MscCa and/or by MS enzymes may modulate the gating of all TRP channels. The physiological and/or pathological effects of this MS modulation remain to be determined. The methods discussed in this chapter, including the application of pressure steps to measure the kinetics of MS enzyme–channel coupling and the use of membrane protein liposome reconstitution for identifying specific protein–lipid interactions should play an increasing role in understanding the importance of the different MS mechanisms underlying TRPC function.

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The role of a mechanosensitive Ca^{2+} channel in regulating prostate tumor cell migration

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ABSTRACT

In its early stages prostate cancer stays in the prostate and is not life-threatening, but without treatment it spreads to other parts of the body and eventually causes death. Because the acquisition of cell motility is a critical step in the metastatic cascade it is important to identify the mechanisms that regulate tumor cell migration. Here we show that the mechanosensitive Ca^{2+} -permeable channel (MscCa) is expressed in human prostate tumor cells and that MscCa activity is required for prostate tumor cell migration. However, MscCa expression alone is not sufficient to confer motility on prostate tumor cells. Instead, MscCa needs to express a specific gating mode and surface distribution in order to coordinate migration. In this case, MscCa and the physical/biochemical mechanisms that regulate its gating and surface distribution should be promising targets to block prostate cancer metastasis.

INTRODUCTION

Prostate cancer (PC) is a progressive disease involving transformation to unlimited cell growth, immortalization to escape the limits of senescence/apoptosis, and the ability to spread to distal sites (invasion and metastasis). In order for PC to spread, tumor cells must migrate from the prostate, pass through blood vessels, penetrate into the secondary tumor site (typically bone), and migrate through its tissue to establish a metastasis (Pienta, and Loberg, 2005). Cell migration is therefore necessary although not sufficient for invasion and metastasis, which also require the additional steps of barrier matrix breakdown, and tumor cell adherence, growth and angiogenesis at the secondary sites (Gupta & Massague, 2006). Nevertheless, because metastasis will only be achieved if the tumor cell completes every step in this cascade, identifying the most sensitive and susceptible step in tumor cell migration should provide a promising target to block PC metastasis (Kassis, et al., 2001).

Although the rates and patterns of cell migration vary among normal and cancer cells (Friedl & Wolf, 2003) they also share a basic cycle of steps involving: 1) the cell's leading edge protruding forward as polymerized actin pushes up against and physically deforms the cell membrane; 2) the front of the cell forming adhesions with the substrate/extracellular matrix (ECM); 3) the cell pulling forward as the myosin-cytoskeleton (CSK) contracts and exerts traction force against the ECM via the cell adhesions; 4) the whole cell becoming progressively stretched as the traction force developed at the cell front pulls against the cell rear; 5) the rear adhesions detaching from

the ECM allowing net cell displacement and relaxation of membrane stretch (Lauffenburger and Horwitz, 1996; Sheetz, et al. 1999; Ridley et al., 2003). A key question about this cycle concerns the mechanosensitive mechanisms that coordinate forward protrusion with rear retraction. Lee and colleagues (1999) proposed from their studies of fast moving fish keratocytes that MscCa could serve this function by its ability to “sense” and transduce membrane stretch into Ca^{2+} influx and thereby provide feedback between mechanisms that cause cell forward protrusion and those Ca^{2+} -dependent mechanisms (e.g., cell contractility and adhesion disassembly) that promote rear retraction. Since the process of cell migration is conserved, we thought that MscCa activity might also be important for coordinating PC cell migration. To test this idea we have studied whether MscCa activity is expressed in PC cells and whether MscCa activity is required for PC cell migration. We also determine whether MscCa expression/properties differ between migratory and nonmigratory PC cells. Previous studies of MscCa have been hampered by the absence of MscCa specific reagents and the lack of identity of MscCa (Lee et al., 1999; Munevar et al., 2004; Doyle and Lee, 2004; Doyle et al., 2004). Here we take advantage of the discovery of a highly specific MscCa blocker, GsMTx-4 (Suchyna, et al., 2004), and the recent identification of the canonical transient receptor potential channel 1 (TRPC1) as a structural subunit of MscCa (Maroto et al., 2005).

RESULTS

MscCa activity in migrating PC cells

Patch-clamp recording and pressure-clamp stimulating techniques were used to study the stretch-sensitive currents in the highly invasive and metastatic human PC cell line, PC-3 (Kaighn et al., 1979; Yang et al., 1999). Figure 1a shows a photomicrograph of a migrating PC-3 cell with its characteristic leading lamellipodium (L), a thicker cell body region (B), and a long extended rear tether (R). Cell-attached patches formed on over 100 PC-3 cells indicate that stretch-activated channels were expressed on all regions of the polarized cell (Fig. 1b) but with a higher percentage of null vs active patches on the lamellipodia (L: 30%, 10 vs 32) compared with either the cell body (B: 18%, 8 vs 46) or the cell rear (R: 12%, 3 vs 25). For these measurements pressure steps were used in order to maximally activate currents in the patch (Fig. 1b, supplementary Figs. 1a & 1c). However, the physiological stimulus during the motility cycle most likely involves a progressive or ramp increase in membrane stretch that lasts for as long as the rate of forward protrusion exceeds the rate of rear retraction. We found that pressure ramps applied to PC-3 cell patches caused a progressive increase in current that did not turn off immediately with the ramp (supplementary Fig. 1b). As discussed below, dramatically different ramp responses are seen in membrane patches from migratory vs nonmigratory PC cells (compare supplementary Figs. 1b & 1d). Fig. 1c indicates that single stretch-activated channels recorded from the same PC-3 cell patch at -50 and 50 mV undergo more frequent gating at negative than at positive potentials. Similar voltage-dependent open channel gating is characteristic of the frog oocyte MscCa (Taglietti and Toselli, 1998), recently shown to be formed by TRPC1 (Maroto et al., 2005). Furthermore, the MscCa current-voltage (I-V) relationships measured in PC-3 cells and frog oocytes

overlap indicating a common pore structure (Fig. 1d, pipette solution contained 100 mM K^+ and 0 Ca^{2+}). Replacing K^+ with Na^+ did not significantly alter the I-Vs (data not shown), whereas including of 1 mM Ca^{2+} reduced inward currents, consistent with permeant Ca^{2+} channel block of the MscCa in both PC-3 cells and oocytes (Fig. 1d) (Taglietti and Toselli, 1998). Significant Ca^{2+} influx via the MscCa under external physiological $[Ca^{2+}]$ is indicated in Fig. 1e, which shows that the stretch-activated inward current (recorded at -10 mV with 100 mM Na^+ and 1 mM Ca^{2+}) was followed by single outward channel currents most likely carried by small conductance Ca^{2+} -activated K^+ (SK) channels (~ 20 pS) since they were blocked by removal of external Ca^{2+} or addition of 50 nM charybdotoxin (data not shown). A “big” conductance Ca^{2+} -activated K^+ (BK) channel (~ 100 pS) that could be activated in all patches, either by depolarization (e.g., ≥ 50 mV) or ionomycin, a Ca^{2+} ionophore, (see supplementary Fig. 2), was not coupled to MscCa activation, indicating that SK, but not BK, may be colocalized with MscCa. We also tested several agents for their ability to block MscCa in PC-3 cells. When 5 μM Gd^{3+} , a relatively nonspecific MS channel blocker (Yang and Sachs, 1989) was backfilled in the pipette solution, MscCa activity was abolished (Fig. 1f and Figs. 2 a & b). A similar effect was achieved with 3 μM GsMTx-4, the most specific MS channel blocker so far identified (Suchyna et al., 2004) (Fig. 1f, and Fig. 2 c & d). We also tested an anti-TRPC1 antibody (Ab) raised against the putative pore region of the TRPC1 channel (Wes et al., 1995; Xu and Beech, 2001). This Ab labeled an 80 kDa protein in PC-3 cell membranes (supplementary Fig. 3a), and when backfilled into the pipette (at 10 $\mu g/ml$), reduced MscCa activity (Fig. 1f). The Ab denatured by heat (100°C for 10 min) in the presence of 10 μM dithiothreitol (DTT) or preincubated with the antigenic peptide had no

effect on MscCa activity. Nor did an anti-TRPC4 Ab raised against the equivalent pore domain region of the hTRPC4 (data not shown).

Anti-MscCa agents block PC cell migration

The same anti-MscCa/TRPC1 treatments were next tested on PC-3 cell migration measured using time-lapse videomicroscopy and wound assays. Figure 3a shows selected video frames (30min apart) of PC-3 cells migrating out from a cluster of 8 cells. Figure 3b (left panels) shows representative PC-3 cell trajectories measured for periods of ≥ 1 h before, during, and after exposure to Gd^{3+} (5 μM), GsMTx-4 (3 μM) and anti-TRPC1 Ab (10 $\mu\text{g/ml}$). The results summarizing several independent experiments are indicated in Fig. 3b (right panels). Apart from blocking directional migration, the agents also altered the cell morphology. For example, instead of a prominent ruffled lamellipodia, the cells took on a smooth, flattened “fried egg” appearance. The effects on migration and cell morphology were rapidly reversed by washing out Gd^{3+} and GsMTx-4, but were irreversible with the Ab (Supplementary video 1). Preincubation of the TRPC1 Ab with the antigenic peptide prevented the block of cell migration and the anti-TRPC4 Ab (10 $\mu\text{g/ml}$) was ineffective in blocking PC-3 migration (data not shown). Figure 3c shows images of PC-3 cell cultures taken at 0, 24 and 48 h during wound assays performed in low serum medium in the absence (control) and the presence of 3 μM GsMTx-4 or 10 $\mu\text{g/ml}$ anti-TRPC1 Ab. The results indicate the failure of wound closure in the presence of GsMTx-4 or the TRPC-1 Ab, consistent with their ability to block PC-3 cell migration. We also transiently transfected PC-3 cells with siRNA targeting TRPC1, which reduced

MscCa activity and TRPC1 expression (Fig 4a, d & e) and blocked migration as measured by time-lapse video microscopy and wound closure assay (Fig. 4b & c). Our results can be compared with a recent study that indicated TRPC1 suppression with siRNA blocked intestinal epithelial cell migration in response to *in vitro* cell wounding, whereas TRPC1 overexpression stimulated cell migration and wound closure (Rao et al., 2006).

$[Ca^{2+}]_i$ dynamics in migrating PC cells

To address how MscCa activity might regulate cell migration we measured the spatial and temporal $[Ca^{2+}]_i$ dynamics in the absence and presence of anti-MscCa agents. Figure 5a shows time-lapse fluorescent images of a migrating PC-3 cell loaded with fura-2, and indicates that as the migrating cell became progressively extended it developed a $[Ca^{2+}]_i$ gradient increasing from the front to the rear of the cell (see also Supplementary Videos 2 and 3). This $[Ca^{2+}]_i$ gradient was a common trait seen in over 200 migrating PC-3 cells. The gradient decreased with rear retraction (step 5) and reversed when cells spontaneously reversed direction (Fig. 5b, supplementary video 4). Some migrating PC-3 cells (~10%) also exhibited fast $[Ca^{2+}]_i$ transients that spread rapidly (≤ 2 min) throughout the cell at an average frequency of 2 ± 0.8 transients/h (range 1-4 transients/h in 15 cells). In some, but not all cases, the transients immediately preceded retraction of the rear tether (see Fig. 5b & c, and supplementary video 4).

GsMTx-4, Gd^{3+} , and the anti-TRPC1 Ab, at the same concentrations previously shown to block cell migration, also prevented the development of $[Ca^{2+}]_i$ gradients and transients in

PC-3 cells (e.g., see Fig. 5d and supplementary video 5 with 10-20 cells tested in each condition). These results are consistent with our observation that the $[Ca^{2+}]_i$ changes were dependent on Ca^{2+} influx, as they were also blocked when 10 mM BAPTA was included in the external solution (supplementary video 6). Similar blocking effects were observed when internal $[Ca^{2+}]_i$ stores were depleted with 5 μ M thapsigargin (data not shown) indicating the $[Ca^{2+}]_i$ dynamics depend upon Ca^{2+} uptake and amplification of Ca^{2+} influx by Ca^{2+} -induced Ca^{2+} release (CICR) and/or Ca^{2+} leakage from internal stores (see also Lee et al., 1999; Braiman & Priel, 2001).

Mechanisms that generate the $[Ca^{2+}]_i$ gradient in migrating PC cells

Several mechanisms may support the spatial $[Ca^{2+}]_i$ gradient that develops in migrating PC-3 and other cell types (see Refs in discussion). In the first case, our patch-clamp studies indicate that MscCa is expressed unevenly on the cell surface with higher density (i.e., fewer null patches) on the cell body and trailing tether compared with the lamellipodia. This was consistent with immunohistochemical studies using fluorescently labeled anti-TRPC1 Ab that indicates a higher density of TRPC1 clusters on the cell body, and particularly on the trailing tether (Fig. 6a & b). In the second case, using fluorescently labeled-thapsigargin (BODIPY FL-thapsigargin) to identify the endoplasmic reticulum (ER) (Gerasimenko et al., 2002) we found a higher density of ER in the cell body than the lamellepodium (Fig. 6c). These results are consistent with sustained and polarized Ca^{2+} influx amplified by CICR from polarized $[Ca^{2+}]_i$ stores generating the $[Ca^{2+}]_i$ gradient seen in migrating cells. Because it has been proposed that $[Ca^{2+}]_i$

gradients may arise from mitochondrial sequestration of fura-2 in some cell types (Quintana and Hoth, 2004), we monitored mitochondrial distribution in living PC3 cells using Mitotracker Red and confocal fluorescence microscopy. Our results indicate that mitochondria were mainly located around the nucleus and in the lamella but were not detectable in the rear tether where $[Ca^{2+}]_i$ becomes elevated (Supplementary Fig 4).

MscCa gating differs in nonmigratory vs migratory PC cell lines

We next asked whether MscCa is expressed in the noninvasive, weakly metastatic PC cell line, LNCaP, originally derived from a PC patient's lymph-node metastases (Horoszewicz et al., 1983; Fischer et al., 2002). LNCaP cells do not develop the polarized morphology of PC-3 cells, but instead show either a spindle or stellar-shaped morphology (Fig 7). Although LNCaP cells do not undergo directional migration they do show pulsating movements as well as multiple mini-lamellipodia and blebs that transiently protrude from around the perimeter of the cell (Supplementary video 7). MscCa is expressed in LNCaP cells and displays a similar single channel I-V relationship as MscCa recorded PC-3 cells (supplementary Fig. 5a). However, MscCa density, surface distribution and gating differ between the two cell lines. First, based on higher average peak currents and the absence of MscCa null patches there is a higher and more uniform surface density of MscCa in LNCaP vs PC-3 cells. This is consistent with Westerns and immunofluorescence experiments using anti-TRPC1 Ab that indicates a higher abundance of TRPC 1 in LNCaP vs PC-3 cells (supplementary Fig. 3). Second, MscCa in LNCaP cells is gated predominately in a transient gating mode (TM) in which channels close rapidly within

~100 ms of a pressure pulse (i.e., in 117 out 135 (87%) patches). This is in marked contrast to the sustained mode (SM) of gating that predominates in PC-3 cell patches (100 out of 118 (85%) of patches) (supplementary Fig. 5b). These different gating modes have profound effects on the amount of charge (i.e., Na^+ and Ca^{2+}) transferred by MscCa, as most dramatically illustrated by comparing their responses to pressure steps and ramps (Figs. 8a & b, see also supplementary Figs. 1b and d). In particular, despite the more than 3-fold larger peak MscCa current activated by pressure steps in LNCaP vs PC-3 cell patches, there was a >20-fold smaller peak current activated by a pressure ramp and an even greater reduction (i.e., >50-fold) in the total charge transferred during the ramp in LNCaP vs PC-3 cell patches. This discrepancy would be expected to increase even more so during the much longer duration ramps of stretch (i.e., > 1 h) experienced by a cell during the migratory cycle.

The TM gating that predominates in LNCaP cells most likely reflects inactivation/desensitization rather than adaptation since closed channels cannot be reopened by simply increasing the stimulus (Fig. 8e) (Hamill and McBride, 1992; Suchyna et al, 1994; Honore et al., 2006; Hamill, 2006). Instead, the stimulus must be turned off and reapplied, indicating the existence of an inactivated state that requires a finite time for recovery (Fig. 8f). MscCa can apparently enter the inactivated state from a closed state (i.e., without opening), which explains why most channels are not available to be activated during ramp stimulation. MscCa inactivation in LNCaP is not strongly voltage dependent (Fig. 8g), but can be lost with mechanical overstimulation of the patch (Hamill and McBride, 1992; Suchyna et al, 1994). For example, repetitive pressure pulses

of 1 s (but not 0.1 s) results in irreversible loss of the transient current without increasing the sustained current (5 out of 5 LNCaP cell patches, Fig 9a). In comparison, in PC-3 cells the SM of gating is relatively robust and shows little run down with repetitive stimulation (Fig 9b).

$[Ca^{2+}]_i$ dynamics in LNCaP cells

We next asked whether nonmigrating LNCaP cells display $[Ca^{2+}]_i$ fluctuations, and if so, whether MscCa plays a role in shaping them. Of the 20 LNCaP cells studied in detail none developed the sustained $[Ca^{2+}]_i$ gradient characteristic of migrating PC-3 cells. The lack of sustained $[Ca^{2+}]_i$ gradients is consistent with the uniform distribution of MscCa and ER/ Ca^{2+} stores (Fig. 7c) as well as the inability of MscCa to transduce stretch into a sustained Ca^{2+} influx. However, 16 out of 20 LNCaP cells did show repetitive $[Ca^{2+}]_i$ transients that spread throughout the cell (Fig. 10a). These transients were faster (≤ 0.5 min) and occurred with a higher frequency (i.e., 8 ± 4.3 transients/h) than the transients observed in some PC-3 cells. Application of GsMTx-4 (3 μ M) inhibited the Ca^{2+} transients/waves (Fig. 10b). The other cells that did not show Ca^{2+} transients displayed localized regions of elevated $[Ca^{2+}]_i$ that were associated with contractile or “tugging” activity at the end of the cell or with membrane protrusive “blebbing” activity (Fig. 10c). These local $[Ca^{2+}]_i$ elevations were absent in LNCaP cells that were incubated in 3 μ M GsMTx-4 (data not shown).

Discussion

Our patch clamp results demonstrate for the first time that MscCa is expressed in the two most commonly studied human PC cell lines — the highly motile and invasive PC-3 line originally isolated from a PC patients bone metastasis and shown to spread and form distal metastases when implanted in nude mice (Kaighn et al., 1979; Yang et al., 1999), and the nonmigratory, noninvasive LNCaP line isolated from a patients lymph node metastasis that only forms local metastases at implantation sites in nude mice (Horoszewicz et al., 1983; Fischer et al., 2002). Our results indicate that MscCa activity is required for PC-3 cell migration and that a difference in MscCa gating and surface distribution seen between PC-3 and LNCaP cells may contribute to their different motilities and metastatic potentials. In particular, in PC-3 cells the MscCa remains gated open during sustained stretch, and MscCa and CICR mechanisms show a polarized distribution with high densities in the rear vs the front of the cell. In contrast, MscCa in LNCaP cell is inactivated rapidly (< 100 ms) by stretch and is uniformly distributed over the cell surface. The third major human PC cell line, DU-145, which was established from a central nervous system metastasis, and like PC-3 cell is highly metastatic, displays the same sustained MscCa gating mode as seen in PC-3 cells (OH and RM unpublished observations).

Single channel current patch clamp measurements are necessarily restricted to the dorsal surface of the crawling cell because it is not possible to patch the ventral “adherent” surface. On this basis it might be argued that since CSK-generated mechanical (traction) forces are transmitted to the substrate at ventral surface adhesions, then only MS channels

localized at these sites should be mechanically activated and play a role in influencing cell migration. However, as traction forces pull on the substrate they will also tend to stretch the whole cell for as long as the rear of the cell remains firmly attached to the substrate (Lee et al., 1999). Apart from the obvious extension of the whole cell (Fig. 1A), other reported manifestations of these stretching forces include the smoothing out of membrane folds and microvilli in spreading cells (Erickson & Trinkaus, 1976), the elastic recoil of some migrating cells when presumably membrane stretch exceeds the strength of rear adhesions (Mandeville & Maxfield, 1997), and in the extreme case even cell rupture/fragmentation that can occur when cell retraction is blocked and membrane stretch exceeds the elastic limits of the bilayer (Verkijovsky et al., 1999). Furthermore, Galbraith and Sheetz (1999) have elegantly addressed the issue of tension distribution on the ventral and dorsal surfaces by using optical tweezers to measure the membrane tension on the dorsal membrane, and a micromachined device to measure tension generated on the ventral membrane. Their measurements demonstrate unequivocally that the dorsal matrix is as effectively linked to the force-generating CSK as the ventral adhesions so that membrane bilayer tension should increase isotropically over the cell surface. As a consequence, MscCa, a bilayer gated MS channel (Zhang et al., 2000; Maroto et al., 2005), will be activated in both dorsal and ventral membrane surfaces of the stretched cell, and MscCa properties as measured with the patch clamp should play a role in shaping $[Ca^{2+}]_i$ dynamics.

The sustained Ca^{2+} influx via MscCa combined with a polarized distribution of MscCa and ER Ca^{2+} stores are well adapted to generating the sustained $[Ca^{2+}]_i$ gradient seen in

migrating PC-3 cells, and may also underlie similar $[Ca^{2+}]_i$ gradients seen in other migrating cell types (Brundage et al., 1991; Gollnick et al., 1991; Hahn et al., 1992; Yumura et al., 1996; Brust-Mascher and Webb, 1998; Schwab et al., 1997; Unterwiesing and Schlatterer, 1995; Kimura et al., 2001; Xu et al., 2004). However, a basic question is how does any cell maintain a sustained $[Ca^{2+}]_i$ gradient over several hours in a cytoplasm that allows free diffusion of Ca^{2+} . In particular, the existence of any stable region of elevated $[Ca^{2+}]_i$ within a continuous aqueous medium would seem to disobey the second law of thermodynamics — according to which solutes should passively diffuse down their concentration gradient until they reach equilibrium, and in the case of Ca^{2+} this equilibration should take only seconds. To explain this apparent paradox, Braiman & Priel (2001) proposed that the cell uses ATP to actively take up Ca^{2+} into internal stores, which is then allowed to passively leak out into localized regions of the cytoplasm. At least consistent with this idea is our finding that blocking Ca^{2+} uptake with thapsigargin abolishes development of the $[Ca^{2+}]_i$ gradient. The interesting aspect of the Braiman & Priel model is that even with uniform Ca^{2+} influx across the cell surface and uniform active $[Ca^{2+}]_i$ uptake, one could still generate a $[Ca^{2+}]_i$ gradient as long as there is polarized Ca^{2+} release sites from otherwise contiguous stores (Petersen et al., 2001). A further possibility is that if both active uptake and passive leak occur within close proximity of the membrane, then a subcortical membrane domain of elevated $[Ca^{2+}]_i$ could be maintained that might go undetected by global $[Ca^{2+}]_i$ measurements.

Another issue related to $[Ca^{2+}]_i$ gradients concerns the possible role played by mitochondria. For example, Quintana & Hoth (2004) have proposed that a $[Ca^{2+}]_i$

gradient seen in T-lymphocytes arises due to fura-2 accumulation in mitochondria, whereas others found that elevated regions of $[Ca^{2+}]_i$ in fibroblasts were not associated with mitochondria but instead colocalized with the Golgi apparatus in the perinuclear region (Wahl et al., 1992). A further complication is that mitochondria are motile, and their motility varies inversely with $[Ca^{2+}]_i$ so that they will tend to move fastest in low $[Ca^{2+}]_i$ (100-300 nM) and slow down or stop in higher $[Ca^{2+}]_i$ (i.e., 1 μ M) (Yi et al., 2004). In this case, one might expect mitochondria to migrate up a $[Ca^{2+}]_i$ gradient and accumulate in regions of highest $[Ca^{2+}]_i$ where they may function as Ca^{2+} buffers to prevent the spread of local $[Ca^{2+}]_i$ transients from regions of high $[Ca^{2+}]_i$ (Tinel et al., 1999; Yi et al., 2004). However, in apparent contradiction of this idea, mitochondria accumulate in the lamella and cell body region rather than in the rear of migrating PC-3 cells and migrating fibroblasts where $[Ca^{2+}]_i$ becomes most elevated (supplementary Fig 4; DeBiasso et al., 1987). The stimulus that promotes mitochondrial accumulation in the lamella remains unclear but could involve the added requirement for ATP and/or an elevated $[Ca^{2+}]_i$ in undetected membrane subdomains.

Functionally, the $[Ca^{2+}]_i$ gradient seen in PC-3 cells would be expected to polarize the expression/activity of Ca^{2+} -dependent molecules (e.g., gelsolin, integrins, calpain, phosphatases, and myosin II) that regulate the processes of protrusion, adhesion and contractility associated with directional locomotion (Hendey & Maxfield, 1993; Arora & McCulloch, 1996; Eddy et al., 2000; Mamoune et al., 2003; Franco & Huttenlocher, 2005). In contrast, in LNCaP cells the rapid inactivation of MscCa and the uniform spatial distribution of MscCa and CICR, should preclude the development of a sustained

$[Ca^{2+}]_i$ gradient. The lack of Ca^{2+} -induced polarization of actin polymerization and adhesion assembly in LNCaP cells may underlie the multiple mini-lamellipodia that protrude transiently and randomly from around the cell's perimeter, in clear contrast with the single prominent and persistent lamellipodium that develops in PC-3 cells (see also Nebl & Fisher, 1997). Although the $[Ca^{2+}]_i$ transients expressed by LNCaP cells are not sufficient to support/generate cell locomotion, a variety of migrating cell types including PC-3 cells express MscCa-dependent $[Ca^{2+}]_i$ transients (Lee et al., 1999; Munevar et al., 2004; Doyle and Lee, 2004; Doyle et al., 2004; Marks and Maxfield, 1990; Giannone et al., 2002; Huang et al., 2004). However, there are different views on how these transients may act to regulate cell movement. One view is that they activate proteins (e.g., calpain and/or myosin II) that promote rear retraction (Lee et al, 1999), while another view is that they are more important in the development of traction forces at the front of the cell (Munevar et al., 2004). In at least some of our PC-3 cell recordings we saw $[Ca^{2+}]_i$ transients that seemed to develop and spread from the rear to the rest of the cell (e.g., Fig. 5a), and on some occasions they immediately preceded rear retraction (see Fig. 5b & c, and supplementary video 4). However, the low frequency (or absence) of $[Ca^{2+}]_i$ transients in PC-3 cells did not prevent their migration nor did their prevalence and high frequency in LNCaP cells promote migration.

Overall, our results indicate that a sustained $[Ca^{2+}]_i$ gradient is a key process in determining migration directionality in PC cells. Further compelling support for this idea comes from a study of migrating cerebellar granule cells in which experimental reversal of the $[Ca^{2+}]_i$ gradient was always accompanied by reversal in migration direction (Xu et al.,

2004). As with PC-3 cells, some granule cells also showed occasional $[Ca^{2+}]_i$ transients, but no causal relationship was noted between the transients and migration direction (Xu et al., 2004). On the other hand, human neutrophils that move by an amoeboid mode of migration rather than a mesenchymal mode do not develop a significant $[Ca^{2+}]_i$ gradient, but do express $[Ca^{2+}]_i$ transients when migrating on adhesive (e.g., fibronectin or vitronectin) but not on nonadhesive substrates (e.g., albumin-coated glass) (Marks & Maxfield, 1990; Hendey & Maxfield, 1993). These transients can be blocked, along with cell migration, by either removing external Ca^{2+} or buffering $[Ca^{2+}]_i$. Under these conditions, the cell appears to be immobilized by its inability to retract its rear, which remains anchored to the substrate, but it is still capable of spreading, assuming a polarized morphology and extending its plasma membrane. In this respect, human neutrophils appear more similar to fish keratocytes that show an increased frequency of $[Ca^{2+}]_i$ transients when becoming stuck on their substrate (Lee et al., 1999). The lack of a sustained $[Ca^{2+}]_i$ gradient in migrating human neutrophils cannot alone be due to their amoeboid mode of migration, since newt “giant” neutrophils and the single cell amoeba (*Dictyostelium discoideum*) develop $[Ca^{2+}]_i$ gradients similar to PC-3 cells (Brundage et al., 1991; Gollnick et al., 1991; Yumura et al., 1996). Presumably, there are several mechanisms that can determine cell polarization and migration directionality.

Our results on PC cells raise the possibility that a shift in Ca^{2+} dynamics mediated by changes in MscCa properties could switch PC cells from a nonmigratory to a migratory mode and vice versa. Previous studies indicate that a shift MscCa gating mode may occur through mechanically-induced changes in interactions between the membrane and CSK.

In particular, it has been shown that either mechanical over stimulation of the patch (Hamill and McBride, 1992; Suchyna et al., 2004) or membrane “blebbing” of the cell (Zhang et al., 2000) can shift MscCa gating from the TM to the SM. Interestingly, Holy (2004) has also recently reported that PC-3 but not LNCaP cells, show spontaneous as well as “cytochalasin-induced” membrane blebbing on their apical surface. This difference would be consistent with why SM gating predominates in PC-3 cells and why mechanical over-stimulation of LNCaP cell patches (which promotes membrane blebbing within the patch) reduces TM gating. These results are also interesting given that during tumor progression the cortical CSK becomes reorganized/more depolymerized (Stournaras et al., 1996) and that Rho-induced membrane blebbing can lead to a switch in tumor cell motility mode (Sahai and Marshall, 2003). Furthermore, several studies have raised the possibility that the changing mechanical environment within a growing tumor including the increasing interstitial stress and fluid pressure, and the increasing tumor stiffness due to perturbed vasculature and fibrosis may promote increased tumor cell motility and escape mechanisms (Sarntinoranont et al., 2003; Paszek et al., 2005).

Other studies indicate that externally applied mechanical forces can switch on cell migration, possibly by acting to increase Ca^{2+} influx pathways. In particular, the application of fluid shear stress or direct mechanical poking of stationary cell fragments formed from fish keratocytes can stimulate fragment polarization and persistent locomotion (Verkhovsky et al., 1989). Similarly, the application of shear stress to endothelial cells (Isshiki et al., 2002) and quiescent *Dictyostelium* can cause CSK reorganization and stimulate cell migration as long as Ca^{2+} is present in the extracellular

bathing fluid (Decave et al., 2003; Fache et al., 2005). One possible explanation is that mechanical shear forces alter the MscCa gating properties (Hamill & McBride, 1992) and/or membrane trafficking of MscCa/TRPC (Maroto et al. 2001; Isshiki et al., 2002; Rizzo et al., 2003; Bezzerides et al., 2004), which in turn alters the $[Ca^{2+}]_i$ dynamics in response to CSK-generated mechanical forces, and this way promotes cell polarization and directional migration. Furthermore, these effects may depend upon membrane raft/caveolae-dependent Ca^{2+} signaling. In particular, Isshiki et al. (2002) found that the caveolae in quiescent endothelial cells are normally clustered around the edge of the cell but when stimulated to migrate, either by wounding a cell monolayer or by exposing the cells to laminar shear stress, the caveolae move to the trailing edge of the cell, concomitant with this relocation the sites of Ca^{2+} waves initiation moving to the same location. In contrast, in human neutrophils lipid rafts and $[Ca^{2+}]_i$ transient initiation sites have been localized to the leading edge of the migrating cell, and cholesterol depletion, which disrupts raft structure, blocks both $[Ca^{2+}]_i$ transient initiation and cell migration (Manes et al., 1999; Kindzelskii et al., 2006). Some insight into these different results may be related to the demonstration that Cav-1, a raft maker, shows a different polarized distribution in endothelial cells depending upon whether the cells are migrating on 2-dimensional substrate or through a 3-dimensional matrix (Parat et al., 2003). In particular, Cave-1 moves from the cell's rear to the cell's front during the switch from the 2-D/mesenchymal to the 3-D/amoeboid migration modes. These findings are highly intriguing given that TRPC1, a structural subunit of MscCa (Maroto et al., 2005), colocalizes with Cave-1 associated membrane lipid rafts (Lockwich et al., 2000; Brasier et al., 2003) and is localized at the leading edge of migrating neutrophils (Kindzelskii et al.,

2004) but in the rear of migrating prostate tumor cells (Fig. 6). Together these results indicate that MscCa may redistribute to different regions of the cell surface and perform different yet critical regulatory functions depending upon the cell's mode of migration. In this case, MscCa may be a preferred therapeutic target to block tumor cell invasion because unlike other regulatory molecules (e.g., metalloproteases, integrins, and calpain) may not become dispensable if the tumor cell switches is able to switch between different migratory modes (Sahai & Marshall, 2003; Wolf & Friedl, 2006; Carragher et al., 2006).

In conclusion, our results indicate that MscCa is a major determinant of $[Ca^{2+}]_i$ dynamics in PC cells and that differences seen between MscCa properties in motile and nonmotile PC cells may be one additional factor that determines the cells invasive phenotype. For this reason there is now added motivation to understand the physical and biochemical mechanisms that regulated MscCa gating and membrane trafficking in order to target these processes to stop tumor cell migration and invasion.

Materials and Methods

Cultures

The human PC cell lines (ATCC, Manassa, VA) studied included the PC-3 (Kaighn et al., 1979), and LNCaP (Horoszewicz et al., 1983). Cell cultures were grown in RPMI 1640 medium with 25 mM Hepes and glutamine, 8 % FCS, 1 mM Na pyruvate, 4.5 g/L glucose and antibiotics at 37°C in a humidified 95% O₂-5% CO₂ atmosphere.

Patch-clamp recording

Standard cell-attached, patch-clamp recording was used to record single-channel currents. A custom-built pressure clamp was used to apply a gentle and reproducible suction protocols (< 10 mmHg for 10 s) to achieve the initial tight seal and then to mechanically stimulate the patch (McBride & Hamill, 1992). The standard pipette solution contained, in mM: 100 KCl, 2 EGTA (KOH), 5 Hepes (KOH) at pH 7.4. To measure Ca^{2+} block and permeation 1 mM Ca^{2+} replaced the 2 mM EGTA. Gd^{3+} was added to the pipette solution without EGTA. The anti-TRPC1 Ab (10-20 $\mu\text{g/ml}$) was included in the bathing solution and/or backfilled into the pipette. In order to monitor MscCa activity before exposure to the agents, the pipette tip was filled (~300 μm) by capillary action with agent-free pipette solution, then backfilled with the agent-containing solution. The standard bath solution contained, in mM: 150 NaCl, 2.5 KCl, 2 CaCl_2 , 1 MgCl_2 and 10 Hepes (NaOH) at pH 7.4. Patch currents were filtered at 500 Hz with an 8-pole Bessel filter and digitized at 1 kHz on an IBM clone using pCLAMP (Axon Instruments, Union City, CA). Chemicals in general were purchased from Sigma (St Louis, MO) except for GsmTx-4 (Peptides International, Louisville, KY). Fluorescent agents from (Invitrogen/Molecular Probes, Carlsbad, CA).

Videomicroscopy and Ca^{2+} -imaging

Cell migration was monitored at 37°C by time-lapse videomicroscopy using Nomarski optics with an Epifluorescent microscope (Nikon). Fura-2 AM (5 μM , 20 min) was loaded for calcium imaging assays. Images were captured at 340 and 380 nm, at 30 s and/or 1 min intervals. Images acquired with Metafluor (Universal Imaging Corp. Sunnyvale, CA)

and a Photometrics Coolsnap HQ camera (Roper Scientific). Metamorph (Version 6.2, Universal Imaging Corp. Sunnyvale, CA) and Excel 2000 (Microsoft Excel, WA) were used for analysis. Conversion of pixels to μm was based on a calibrated slide. Wound assays were carried on sub confluent PC3 cells (90%) seeded on 35 mm dishes. Three scratches ($\sim 500\ \mu\text{m}$ across) per dish were made using a sterile 200 μl pipette tip. As indicated, 3 μM GsMTx-4 or 10 $\mu\text{g/ml}$ anti-TRPC1 Ab were added to the culture and replaced by fresh solution after 24 h. The same procedure was used in controls.

Immunofluorescence

Cells were incubated (30 min, 37° C) with anti-TRPC-1 Ab (10 $\mu\text{g/ml}$) raised to the external pore region of TRPC1, diluted in MR. The secondary antibody (2.5 $\mu\text{g/ml}$, goat anti-rabbit ALEXAfluor 568) was applied 30 min at RT, then washed out and cells fixed (2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% picric acid, 0.03% CaCl_2 and 0.05M cacodylate buffer pH 7.4). In control experiments, a single incubation with the secondary antibody was performed. A Zeiss LSM 510 META confocal system configured on an Axiovert 200M inverted microscope (63X 1.4 objective) was used to acquire the images (543nm excitation, green He/Ne laser) later processed with Metamorph. The distribution of ER in PC cells was studied by 10 min incubation with 200 nM BODIPY FL-thapsigargin (excitation 488, emission 510-600).

Westerns

Cell lysates collected in cold buffer (mM: 10 Tris, 150 NaCl, 5 EDTA, 1 PMSF; 1M KI, 2.5 $\mu\text{l/ml}$ protease inhibitor cocktail, Sigma, and 1.5% OG) were shaken 15 min at 4°C

and centrifuged (14,000 RPM, 4°C, 30 min). The supernatants stored at -80°C, and protein concentrations determined by bicinchonic acid assay (BIO-RAD, Hercules, CA). Samples were resolved in an 8% SDS/PAGE gel, transferred to a PVDF membrane, and developed by ECL. Blots were incubated overnight with the primary antibody in the cold room. An anti-TRPC1 Ab was generated against the T1e3 epitope (CVGIFCEQQSNDTFHSFIGT) that lies between the S5 and S6 membrane spanning domains, the proposed pore region of the channel (Wes et al., 1995; Xu and Beech, 2001). We also generated an anti-TRPC4 Ab against the equivalent T1e3 epitope of TRPC4 (CKGIRCEKQNNAFSTLFETQ). The reactivity of the anti-TRPC1 Ab to a 80 kDa protein was identical to that of a commercial anti-TRPC1 Ab raised against the peptide QLYDK GYTSK EQKDC (amino acid residues 557-571 intracellular terminus of hTRPC1, accession# P48995, lot# AN-02 Alomone Labs, Jerusalem, Israel). The Ab specificity for the 80 kDa protein was tested with the antigenic (control) peptides. The anti-TRPC4 Ab reacted with a 110 kDa protein (which was detected in LNCaP cells but not in PC-3 cells) similar to that identified by the commercial anti-TRPC4 Ab raised against the peptide corresponding to residues 943-958 of the C-terminus of mTRPC4 (lot# AN-01 Alomone Labs). The anti-TRPC1 Ab was also tested under denaturing conditions (10 min, 100°C and 10 μ M DTT).

siRNA experiments

PC-3 cells were transfected with 25 and 50 nM of double stranded siRNA Cy3 labeled (validated to knock down TRPC1 expression by ~90% in 48 h, ID# 7311) or a negative control (scrambled) (Ambion, Austin, TX), using Lipofectamine 2000 (Invitrogen,

Carlsbad, CA) according to the manufacture instructions. Fluorescently labeled cells (positive and negative control) were tested after 48-72 h .

SYBR-Green RT-PCR

Total RNA was isolated from PC-3 pelleted cells using RNAqueous Midi (Ambion, Austin, TX). RNA samples were quantified using a Nanodrop Spectrophotometer and qualified by analysis on an RNA Nano-chip using the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA was synthesized using Taqman Reverse Transcription Reagents Kit (ABI #N8080234) at the conditions (min): 10, 25°C; 30, 48°C and 5, 95°C. The Q-PCR amplifications were performed in triplicate using SYBR Green PCR Master Mix (ABI #4364344). The TRPC-1 primer pairs were designed to cross an intron junction (TCAAAGGAGCAGAAGGACTGTGTAG, forward GGTGCCAATGAACGAATGG, reverse), final concentration 900 nM. Relative RT-QPCR assays were performed with 18S RNA. PCR assays were run in the ABI Prism 7000 Sequence Detection System at the conditions (min): 2, 50°C; 10, 95°C. Cycling was performed 40 times 15sec at 95°C and 1 min at 60°C.

Online supplemental material

There are five supplementary figures and seven supplementary videos. Supplementary (Suppl) Figure 1 compares MscCa responses to pressure steps and ramps in PC-3 and LNCaP cells. Suppl Figure 2 shows two types of Ca^{2+} -activated K^{+} channels (BK and SK) in PC cells. Suppl Figure 3 compares TRPC1 expression measured in PC-3 and LNCaP cells as well as immunofluorescent detection of TRPC1 in LNCaP cells. Suppl

Figure 4 shows the distribution of mitochondria in the front or nuclear region and not in the rear of the cell where the $[Ca^{2+}]_i$ was the most elevated. Suppl Figure 5 compares MscCa current-voltage relations and the amplitude and gating characteristics in PC-3 and LNCaP patches. Video 1 shows the effects of GsmTX-4 and anti-TRPC1 antibody of PC-3 cell migration. Video 2 shows the development of a $[Ca^{2+}]_i$ gradients in a migrating PC-3 cell. Video 3 shows $[Ca^{2+}]_i$ changes in a group of PC-3 cells as they migrate out of a cluster. Video 4 shows a reversal of $[Ca^{2+}]_i$ gradient that is accompanied by reversal in migration direction. Video 5 shows reversible block by GsmTx-4 of $[Ca^{2+}]_i$ dynamics and cell migration. Video 6 shows the effects of chelation of external Ca^{2+} by BAPTA on $[Ca^{2+}]_i$ and PC-3 cell migration. Video 7 is time-lapse recording of LNCaP cells showing multiple random lamellipodia without directional movement.

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ABBREVIATIONS LIST

CSK, cytoskeleton; ECM, extracellular matrix; GsmTX-4 *Grammstola* spider venom peptide 4 kDa; MscCa, mechanosensitive Ca^{2+} -permeant channel; PC, prostate cancer; TRPC1, canonical transient receptor potential channel 1.

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Figure legends

Figure 1. MscCa properties in the human prostate tumor cell line, PC-3. **a:** Photomicrograph of a PC-3 cell indicating three distinct morphological regions: the leading lamellipodium (L), the cell body region (B), and the rear tether (R). **b:** Representative cell-attached patch recordings made from the indicated region of different PC-3 cells. The mean amplitude of the MscCa currents excluding null patches from each region were similar: L, 23.5 ± 3.58 pA ($n = 22$); B, 24.5 ± 2.56 pA ($n = 38$); and R, 26.0 ± 3.8173 ($n = 22$) whereas the percentage of null patches was 30% (L); 18% (B) and 12% (R) **c:** Suction step applied to a cell-attached patch held at -50 mV and 50 mV activates single channel currents with more frequent open channel gating events at the negative potential. **d:** Single-channel current-voltage relationship of the MscCa measured on cell-attached patches of PC-3 cells (solid symbols) and *Xenopus* oocytes (hollow symbols) with zero Ca^{2+} (circles, pipette solution in mM: 100 KCl, 5 Hepes, 2 EGTA) and 1 mM Ca^{2+} (triangles, pipette solution in mM 100 NaCl, 5 Hepes 1, CaCl_2) (data based on 4-10 patches for each cell type and each ionic condition). **e:** Pressure activation of an inward MscCa current results in a delayed activation of single outward channel currents (Pipette

solution; 100 NaCl, 5 Hepes and 1 mM CaCl₂, patch potential: ~ -10 mV). **f:** Histogram showing the % of patches with MscCa activity in the absence and presence of anti-MscCa agents (5 μ M Gd³⁺, 3 μ M GsMTx-4 or 20 μ g/ml anti-TRPC1 Ab) in the pipette solution. (***) $p < 0.001$).

Figure 2. The Gd³⁺ and GsMTx-4 block of MscCa in PC-3 cell patches. Top panel: Cell-attached patch recordings showing the patch current in response to increasing steps of pressure measured soon after forming the tight seal (~10 s) (**a**) and ~3 min later (**b**). In this experiment the pipette tip (~300 μ m from the orifice) initially contained Gd³⁺-free solution and the rest of the pipette contained 5 μ M Gd³⁺ solution. The 3 minute time interval ensured that Gd³⁺ had diffused to the patch as measured previously with dye. Lower panel: A similar protocol showing patch currents before (**c**) and after (**d**) block by 3 μ M GsMTx-4.

Figure 3. PC-3 cell migration and the effects of anti-MscCa agents. **a:** Selected video frames 30 min apart showing PC3 cells migrating out of the cluster. **b:** Left panels show representative trajectories (monitored every 5 minutes) before, during, and after application of 5 μ M Gd³⁺, 3 μ M GsMTx-4 and 10 μ g/ml anti-TRPC1 antibody. Right panels show histograms from 25 or more cells (mean \pm SEM). See also supplementary video 1 showing reversible block of migration by GsMTx-4 and irreversible block by anti-TRPC1 Ab (20X magnification, NA 0.75). **c:** Photomicrographs (10X magnification Ph NA 0.25) of migrating PC3 cells after wounding in low serum medium (control) and

blocking effect of the addition of GsmTx-4 or anti-TRPC1 Ab, at the same concentrations mentioned above.

Figure 4. MscCa activity, cell migration and TRPC1 expression in PC-3 cells transfected with siRNA targeting TRPC1. **a:** Histogram of MscCa activity in cell-attached patches on PC-3 cells transfected with siRNA targeting TRPC1 vs scrambled RNA. All recordings were made at -50 mV patch potential. **b:** Histogram summarizing the results recorded from time-lapse experiments performed in scrambled and siRNA transfected PC3 cells. A significant inhibition of cell migration was monitored at any interval of time in TRPC1 knocked down PC3 cells compared with the scrambled population. **c:** Photomicrographs of migrating wild type and scrambled PC3 cells after wounding, maintained in 8% FCS RPMI culture medium, show a similar time closure while the siRNA TRPC1 population did not reach the healing after 24 h. Pictures were taken using 10X magnification. **d:** Western blot showing TRPC1 expression in PC3 cells transfected with siRNA and scrambled RNA. The amount of total protein loaded per lane was 60 μ g. **e:** The results from RT-PCR assays indicate a decrease in mRNA transcripts in PC-3 cells transfected with siRNA compared with wild type and PC-3 cells transfected with scrambled RNA (average of three experiments).

Figure 5. Two types of $[Ca^{2+}]_i$ fluctuations in migrating PC-3 cells. **a:** $[Ca^{2+}]_i$ fluorescent images from left to right of a locomoting PC-3 showing regions of high $[Ca^{2+}]_i$ that develop initially in the rear half of the cell and spread to the front of the cell. This particular cell was monitored for 3.5 h (see Supplementary video 2). **b:** $[Ca^{2+}]_i$ images of

two PC-3 cell initially migrating in opposite directions and with opposite $[Ca^{2+}]_i$ gradients (30 min frame) (arrows). In the next frame (125 min) cell 1 had reversed its $[Ca^{2+}]_i$ gradient and migration direction. This cell then showed a Ca^{2+} transient (136 min frame) that was followed by retraction of its tether (145 min) and the cell continued moving (186 min). The original recording was made over 4.8 h. (see supplementary video 4) **c:** Fast Ca^{2+} transients in a migrating PC-3 cell. From left to right, Ca^{2+} images of two PC-3 cells in which the migrating cell 1 showed $[Ca^{2+}]_i$ transients while the stationary cell 2 did not (see also supplementary video 4). For all above images 100X 1.3 NA objective was used. **d:** GsMTx-4 reversibly reduced $[Ca^{2+}]_i$ elevations in PC-3 cells. Three $[Ca^{2+}]_i$ images showing PC-3 cells before, after 5 min exposure to 3 μ M GsmTx-4 solution and 30 minutes following GsmTx-4 washout in which the $[Ca^{2+}]_i$ had recovered to levels above those before GsMTx-4 exposure. Images taken with a 20X 0.75 objective.

Figure 6. Two mechanisms that may sustain the $[Ca^{2+}]_i$ gradient seen in migrating PC-3 cells. **a & b:** Transmission and immunofluorescent confocal images of two PC-3 cell labeled with anti-TRPC1 Ab indicates a higher density of TRPC1 on the rear compared with the front of the PC-3 cells. **c:** Transmission and immunofluorescent confocal images of a PC-3 cell labeled with 200nM BODIPY FL-thapsigargin indicates a higher distribution of the ER/internal Ca^{2+} stores on the cell body compared with the lamellipodium. Fluorescent images represent the maximum intensity projection reconstructed from a stack of 25 confocal sections obtained at 0.2 μ m intervals.

Figure 7. Photomicrographs showing LNCaP cell morphologies. A stellar-shaped LNCaP cell (**a**) and a bipolar LNCaP cell (**b**). Although LNCaP cells do not undergo directional locomotion, they did show random pulsating movements. In bipolar cells protrusive/contractile activities at the ends of the cell could produce extension and thinning of the cell without net displacement. **c.** Confocal images of an LNCaP cell incubated with BODIPY FL-thapsigargin to determine the ER distribution. The fluorescent image represents the maximum intensity projection reconstructed from a stack of 30 confocal sections obtained at 0.2 μm intervals. Both transmission and fluorescent images overlapped indicating an almost uniform distribution within the cytoplasm.

Figure 8. Comparison of MscCa gating in LNCaP and PC-3 cells. **a:** The current response of a cell-attached patch on an LNCaP cell to a suction step of 60 mmHg showed a transient current of approximately 140 pA indicating ~70 channels in the patch that inactivated within 100 ms of the step. **b:** the response of the same patch to a ramp of 100 mmHg activated only 1-2 channel currents at the beginning of the ramp and a smaller noiser currents of only 2-4 pA by the end of the ramp. In this experiment reapplication of the step shown in Fig. 1 a reactivated ~130 pA indicating the channels had not run down. **c:** The current response of a cell-attached patch on a PC-3 cell to a suction step of 60 mmHg showed a transient current of approximately 40 pA indicating ~20 channels in the patch that remained open during the step and showed only delayed closing after the step **d:** The response of the same patch to a ramps of 100 mmHg showing the activation of peak currents of 40-50 pA that underwent a delayed decline after the ramps. **e:** A double step suction protocol failed to activate a significant increase in current even though the

initial current was not saturating. **f:** On the same patch as in e application of a second step after the first steps activated a larger current. The patch responses in e & f are indicative that the transient gating represents tension-induced inactivation rather than adaptation since all the channels do not need to open to enter the inactive state and require a finite time to recover from the inactivated state before they can be opened. **g:** The inactivation of the current seen in LNCaP cells is not strongly voltage dependent in contrast to the adaptation-like behavior reported in the oocyte (Hamill & McBride, 1992).

Figure 9. MscCa in LNCaP cells can transduce repetitive brief suction steps but shows run down with longer suction steps. **a.** Patch response to repetitive 100 ms suction steps. Examination of the responses at higher resolution indicated many channels (> 50%) had closed within the first 50 ms. The constant amplitude responses indicate high fidelity in transducing brief stimuli. **b.** A different patch that shows a run down of the transient gating in response to repetitive 1 s suction pulses. Prior to this recording the patch was stimulated with 20 pulses with little reduction in the peak current. Note the small component of SM gating was relatively constant throughout. The run down of MscCa appeared irreversible in that it did not recover after several minutes without stimulation. **c.** Another patch formed on a PC-3 cell showing that immediately after forming a gentle seal the MscCa was present in the sustained mode and did not show run down with repetitive 1 s suction steps.

Figure 10. The LNCaP shows Ca^{2+} transients that are blocked by GsmTX-4 **a.** $[\text{Ca}^{2+}]_i$ imaging of an LNCaP cell showing two Ca^{2+} transients at 20 min and at 22-23 min after

beginning the recording. The lower graph is a line scan of $[Ca^{2+}]_i$ levels (340/380 ratio) in the same cell showing two bursts of $[Ca^{2+}]_i$ transients. **b.** Line scan on another LNCaP cell in which 3 μ M GsmTx-4 was applied (dashed line), causing a reversible decrease in the $[Ca^{2+}]_i$, as well as blocking the Ca^{2+} transients. **c.** $[Ca^{2+}]_i$ imaging of an LNCaP cell where the arrows indicate the fast and reversible formation of protrusions accompanied by transient local $[Ca^{2+}]_i$ elevations.

Online Supplemental Material

Supplementary figure 1. Comparison of cell-attached patch responses in human PC cells to suction steps and ramps. **a:** The responses of a PC-3 cell patch stimulated first with a series of steps then a series of ramps showing the sustained opening of MscCa during stimulation and the delayed closure after stimulus turn off. **b:** The responses of a LNCaP cell patch to a series of steps and then ramps showing the rapid closure of MscCa within 200 ms of the step and the relatively small responses to the ramps.

Supplementary figure 2. Ca^{2+} -activated big conductance (BK) and small conductance (SK) K^+ channels in PC-3 cells. **a:** Cell-attached patch recording on PC-3 cell patch bathed a Krebs solution (i.e., with 1.8 mM Ca^{2+}) containing 5 μ M ionomycin. The currents were recorded at approx. -10 mV with 100 mM NaCl and 1 mM $CaCl_2$ in the pipette solution. **b:** Current-voltage relation of the BK and SK channels measured in the same patch.

Supplementary figure 3. Comparison of TRPC1 protein expression and functional MscCa expression in PC-3 and LNCaP cells. **a.** Westerns blots showing the expression of an ~80 kD band labeled by an anti-TRPC1 antibody against the external pore region of the channel²² (see methods) with different amounts (10, 20 and 40 μ g) of PC-3 and LNCaP membranes in the absence and presence of the antigenic/blocking peptide (bp). **b.** Immunofluorescent image of an LNCaP cell incubated with anti-TRPC1-Ab and ALEXA fluor 568 shows the relative homogeneous distribution of TRPC1 over the LNCaP cell body compared with the polarized surface distribution seen PC-3 cells (see Figs. 6a & b)

Supplementary figure 4. Distribution of mitochondria in living PC3 cells. **a.** Confocal image of a PC3 cell loaded with Mitotracker-Red (100 nM) to visualize the mitochondria distribution. The fluorescent image represent the maximum intensity projection reconstructed from a stack of 20 confocal sections obtained at 0.2 μ m interval using a 63X1.4 objective (excitation 581, emission 644). DAPI (emission 457) was simultaneously applied to visualize the nuclei. **b.** Fluorescent image of a migrating PC3 cell after incubation with Fura 2 (left panel) and Mitotracker-Red (central panel) shows a high $[Ca^{2+}]_i$ in the tether of the cell while the mitochondria is surrounding the nucleus. PC3 cells (14 out of 16 cells tested shown the same pattern). Images acquired under 100X1.3 magnification.

Supplementary figure 5. Comparison of I-V relations, patch density and gating mode of MscCa recorded in LNCaP and PC-3 cells. **a.** The single channel I-V relations for MscCa measured on cell-attached patches of LNCaP and PC-3 cells measured with 100

mM KCl 2 EGTA (KOH) and 5 mM Hepes (KOH) in the pipette solution. Data points based on 10-20 patches for each PC cell type. **b.** Histograms showing the MscCa peak currents and the percentage of patches showing SM and TM gating in LNCaP and PC-3 cells. For PC-3 cells, 135 patches with MscCa activity were studied, and 117 showed SM gating with the remainder showing some evidence of TM gating (i.e., rapid current inactivation at the beginning of the pulse with a residual current). For LNCaP cells, of the 118 patches studied 100 showed TM gating in which nearly all channels (>90%) closed within ~100 ms of the step.

Supplementary video 1: Time-lapse videos showing two groups of PC-3 cells migrating out from cell clusters before, during and after application of 3 μ M GsMTx-4 (left panel) and 10 μ g/ml anti-TRPC1 Ab. Before adding the agents the cells spread wide ruffled lamellas while locomoting. In the presence of the agents the cells lose their polarized morphology and stop migrating. The blocking effect was rapidly reversed with washout of GsMTx-4 but persisted after washout of the Ab. Frames were saved at 5 min intervals using Nomarski optics under 20X 0.75 magnification. Displaying rate is 10 frames/sec.

Supplementary video 2: Time-lapse $[Ca^{2+}]_i$ imaging of a migrating PC-3 cell (selected frames are shown in Fig. 3a) showing the development of a $[Ca^{2+}]_i$ gradient (front-low/rear-high) as the lamellipodium protrudes forward and the rear becomes extended. The rear tether was not retracted before the end of this recording, which lasted 3.5 h. In the very last frames a transient rise in $[Ca^{2+}]_i$ developed in the very cell rear and spread

incompletely towards the cell front. Frames recorded at 1 min intervals using 100X 1.3 magnification. Displaying rate is 10 frames/sec. See Fig 5a.

Supplementary Video 3 legend.

Time-lapse $[Ca^{2+}]_i$ imaging of a group of PC-3 cells as they migrate out from a cell cluster. All cells showed evidence of a $[Ca^{2+}]_i$ gradient (increasing from front to rear) opposite to their direction of migration. The original recording was made over a 2 h 53 min period and images were captured at 1 min frame intervals using 40X 0.9 magnification. Displaying rate is 10 frames/sec.

Supplementary Video 4 legend.

Time-lapse $[Ca^{2+}]_i$ imaging of two PC-3 cell with initially opposite $[Ca^{2+}]_i$ gradients and migrating in opposite directions (Selected frames are shown in Fig. 3b). As the cell in the upper right changed its direction it retracted one tether on the left, and there was a decrease in local $[Ca^{2+}]_i$ at the retraction site. The same cell then developed another tether that was retracted almost immediately after a Ca^{2+} transient spread throughout the cell. The same cell then underwent a reversal in the $[Ca^{2+}]_i$ gradient as the cell spontaneously changed direction. The original recording was made over 4.8 h. using 100X 1.3 magnification. Displaying rate is 10 frames/sec. See Fig. 5b.

Supplementary Video 5 legend.

Time-lapse $[Ca^{2+}]_i$ imaging of a group of PC-3 cells recorded as they migrate out of the cluster and develop Ca^{2+} gradients. Both migration and $[Ca^{2+}]_i$ fluctuations, were

reversibly blocked in the presence of 3 μ M GsMTx-4. Frames were taken every minute under 40X 0.9 magnification. Displaying rate is 10 frames/sec.

Supplementary video 6 legend

Time-lapse $[\text{Ca}^{2+}]_i$ imaging of a group of PC-3 cells showing the blocking effect of 10 mM extracellular BAPTA on cell migration and $[\text{Ca}^{2+}]_i$ gradients. Frames were taken every minute under 40X 0.9 magnification. Displaying rate is 10 frames/sec.

Supplementary video 7 legend

Time-lapse recording of two LNCaP cells to illustrate the morphological changes observed as small lamellas quickly form, spread and disappear without promoting net cell migration. Frames were taken using Nomarski optics under 100X 1.3 magnification. Displaying rate is 10 frames/sec.

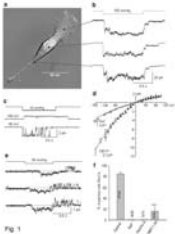


Fig. 1

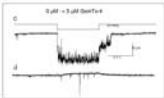
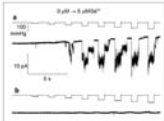
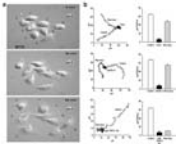
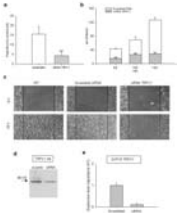


Fig. 2





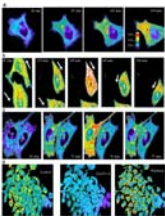


Fig 5

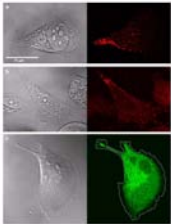


Fig. 8



Fig 7

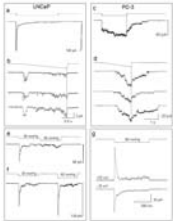
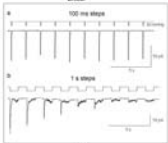


Fig. 8

LACaP



PC-8

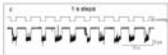


Fig 9

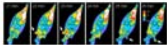
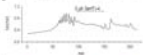
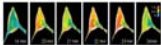
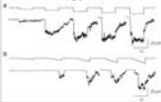


Fig. 10

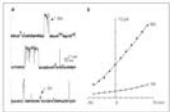
PC-3



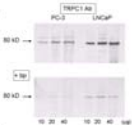
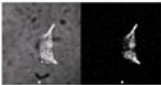
LNCaP



Supplementary Fig. 1



Supplementary Fig. 2

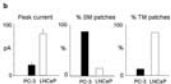
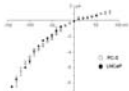
a**b**

Supplementary Fig 3



Figure 6. Fluorescence images of a cell. (a) Fluorescence image showing a cell with a red nucleus and a cyan cytoplasm. (b) DIC image of the same cell. Scale bar: 10 μ m.

Figure 7. Fluorescence images of a cell. (a) Fluorescence image showing a cell with a yellow nucleus and a green cytoplasm. (b) Fluorescence image showing a cell with a red nucleus. (c) DIC image of the same cell.



Supplementary Fig 1

Revisiting TRPC1 and TRPC6 mechanosensitivity

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Abstract

This article addresses whether TRPC1 or TRPC6 are essential components of a mammalian stretch-activated mechano-sensitive Ca^{2+} permeable cation channel (MscCa). We have transiently expressed TRPC1 and TRPC6 in COS or CHO cells and monitored the activity of the stretch-activated channels using a fast pressure clamp system. Although both TRPC1 and TRPC6 are highly expressed at the protein level, the amplitude of the mechano-sensitive current is not significantly altered by overexpression of these subunits. In conclusion, although several TRPC channel members, including TRPC1 and TRPC6, have been recently proposed to form MscCa in vertebrate cells, the functional expression of these TRPC subunits in heterologous systems remains problematic.

Introduction

MscCa, also referred to as a stretch-activated cation channel (SAC), was recognized more than 20 years ago during patch clamp studies of chick skeletal muscle, and shown to be present in most, if not all, eukaryotic cells (8, 9). MscCa displays a range of permeability properties indicating a heterogeneous composition. Initially, MscCa was proposed to derive its stretch sensitivity from the cytoskeleton (CSK)(8), but MscCa activity in CSK-deficient membrane vesicles and liposomes indicates the channel can also be gated by forces within the bilayer (18, 29). A membrane protein solubilization/reconstitution procedure similar to that used to identify MscL, a prokaryotic mechano-sensitive channel (25), was used to identify a *Xenopus* membrane protein fraction that reconstituted MscCa activity and was abundant in a ~80 kDa protein fraction (18). Immunological methods demonstrated TRPC1 presence in the active fraction (18). Furthermore, heterologous expression of hTRPC1 increased MscCa activity, while anti-sense

hTRPC1 reduced endogenous/background oocyte channel activity (18). Overexpression of hTRPC1 was also reported to increase MscCa activity in Chinese hamster ovary (CHO) cells (18).

However, several recent developments warrant a novel study and discussion. First, a TRPC1^{-/-} knock-out mouse has been generated that shows no apparent phenotype (6). This work further demonstrates that TRPC1 is not a store-operated channel (SOC) (6). Second, mammalian cell lines can display endogenous MscCa activity similar to that associated with hTRPC1 overexpression (18), and third, a closely related TRPC family member, TRPC6, has been reported to function as MscCa (23). Additionally, TRPA1 has also been implicated in mechanosensation of the nematode *C. elegans* and was proposed as a candidate mechanosensor in mammalian hearing, although knock out studies in the mouse failed to confirm this hypothesis (4, 5, 14).

In this report, we have studied the functional expression of TRPC1 and TRPC6 in both transiently transfected CHO and COS cells in comparison with the mechano-gated K_{2P} channel TREK-1 (12). Our study demonstrates that the functional expression of both TRPC1 or TRPC6 is problematic, thus leaving open the question for a specific role of these subunits in MscCa activity.

Results

Expression of TRPC1 and TREK-1 in transiently transfected COS cells

Although the results presented by Maroto and colleagues were promising (18), the reported ~ 10-fold increase in MscCa activity seen with expression of hTRPC1 was much less than the 1000-fold increase achieved with overexpression of other channels such as the mechano-sensitive K_{2P} channel TREK-1 (Fig. 1a-b). Furthermore, data presented here, and representing a much larger sample than originally studied, indicate that control COS-7 can express levels of background

MscCa activity that is as high as that reported in hTRPC1-transfected cells in CHO cells (18)(Fig. 1c-d). These channels are cationic non-selective with a conductance of 28.8 ± 0.3 pS ($n = 11$) (Fig. 2). The mean peak current amplitude was 9.71 ± 1.34 pA ($n = 70$) and 10.23 ± 0.99 pA ($n = 140$) for hTRPC1 and empty pIRES2 EGFP expressing cells, respectively. At the 0.05 level, the means are not significantly different (one-way ANOVA). After removing the silent patches (with no channel activity at -80 mm Hg), the mean peak current amplitude became 13.59 ± 1.46 pA ($n = 50$) and 14.77 ± 1.16 pA ($n = 97$) for hTRPC1 and empty pIRES2 EGFP expressing cells, respectively. Again, at the 0.05 level, the means are not significantly different (one-way ANOVA). Moreover, the background MscCa activity is not stable and varies from cell-to-cell within the same culture and from experiment-to-experiment (Fig. 3). The basis of this variability and whether it arises through heterogeneities in endogenous TRP channel expression remains to be determined. When investigated at different pressures over a range of 80 mm Hg, no significant difference was found between the TRPC1 and the empty expression vector expressing cells, unlike the TREK-1 expressing cells (20) (Fig. 4).

Is TRPC1 expressed at the plasma membrane?

An issue concerns the proportion of expressed hTRPC1 that is inserted in the plasma membrane (11). Unlike with TREK-1 (Fig. 5a) and in agreement with previous studies (11), most of the expressed hTRPC1 fails to reach the plasma membrane of mammalian cells including COS and CHO cells but instead accumulates in the endoplasmic reticulum (Fig. 5b, 5d)(Supp movies 1 and 2). TRPC1 was distributed throughout the cell and not obviously located at the cell membrane in both CHO and COS cells (Fig. 5b, 5d)(Supp movies 1 and 2). In comparison, hTRPC1 expressed in frog oocytes was apparently concentrated in the surface membrane (Fig. 5c).

Expression of TRPC1 in transiently transfected CHO cells

For CHO cells (Fig. 6), over a period of one year, we observed a random variation of three orders of magnitude in background stretch-activated currents (Fig. 6 black squares). CHO cells transfected with hTRPC1 had the same basic response (Fig. 6 red circles). hTRPC1 transfected and untransfected cells are indistinguishable. The unitary conductance was $(34 \pm 2.6 \text{ pS})$, similar to that reported by Maroto et al. and the reversal potential was around 0 mV (18)(not shown). The data of Maroto et al., obtained in CHO cells are indicated in Figure 6 as blue asterisks and they fall in the same range of current amplitude as those in non-transfected control cells observed in the present study (18). Therefore, we cannot conclude that an increase in a stretch-activated current in these mammalian cells was due to the expression of the cloned hTRPC1 channel.

Expression of TRPC6 in transiently transfected COS cells

Membrane insertion of TRPC1 can be increased by co-transfection with other TRPCs (11) suggesting that endogenous TRPCs may combine with hTRPC1 to form heteromeric channels (11, 24, 26). As a consequence, any variation in endogenous TRPC expression with clone, passage number and/or culture condition could influence the level of expressed as well as endogenous channel activity. TRPC6 is of particular interest because recent results indicate that hTRPC6 expression in CHO cells also leads to increased MscCa activity (23). This would be consistent with the proposal that TRPC6 participates in the pressure-dependent myogenic contraction of cerebral arteries (27), and indeed, antisense oligonucleotides to TRPC6 attenuate the arterial smooth muscle depolarization and constriction caused by elevated intraluminal pressure (27). However, an indirect mechanism involving diacylglycerol activation following phospholipase

C stimulation has initially been proposed to account for the role of TRPC6 in the arterial myogenic contraction (27).

We have transiently transfected TRPC6 into COS cells and examined the amplitude of the stretch-activated current (Fig. 7). Preliminary experiments demonstrated that TRPC6 was highly expressed at the protein level upon transfection (Fig. 7a). However, the amplitude of the mechano-sensitive currents recorded in the cell-attached patch configuration was not different between the mock-transfected and the TRPC6-transfected cells (Fig. 7b-c), although intracellular OAG application (100 μ M) consistently increased channel activity, in agreement with previous reports (10). Again, as previously observed for TRPC1, a large variability was observed within each transfection (Fig. 7d-e).

Discussion

All together these results fail to confirm a significant role for TRPC1 or TRPC6 in stretch-activated channels when expressed in either COS or CHO cells (18, 23). However, in the same experiments, expression of the K_{2P} channel TREK-1 yields reproducible large amplitude stretch-activated K^+ currents as previously described (13, 20).

Stretch-sensitivity of a channel in the patch does not prove the channel functions as a mechanotransducer either under physiological or pathological conditions. One also needs to show that modifying channel activity/expression can affect a mechanically-sensitive process (3, 17). However, TRPC6^{-/-} mice actually show increased rather than decreased myogenic tone, and are hypertensive (7). This unexpected phenotype has been interpreted as arising from upregulation of TRPC3 that serves a similar function as TRPC6 (7). The results of a TRPC1^{-/-} knock out mouse are even more puzzling since this animal shows no

phenotype, and develops normally even though TRPC1 is the most widely expressed TRPC subunit and has been implicated to be an essential component of the store-operated channel (6). Perhaps less surprising, because of the role of TRPC6, TRPC1 deletion does not affect vascular mechanotransduction, nor does it lead to any detectable upregulation of other TRPCs (6). These results may indicate a normal redundancy of TRPC channels within cells in which several channels perform similar functions (2). An analogous situation seems to apply in *E.coli* where knock out of MscL alone produces no phenotype, and only when MscS is also deleted, do the cells show abnormal growth in response to osmotic stress (16). Similar redundant mechanisms in vertebrate cells may compensate when specific MscCa activity is blocked during *Xenopus* development (28). Clearly, the situation with MscCa contrasts with that seen with the stretch-activated K⁺ channels encoded by the K_{2P} channel subunits where TREK-1 expression in either *Xenopus* oocytes or COS-7 cells results in robust mechanosensitive K⁺ currents in excised patches (≥ 1 nA ; Fig. 1a-b)(12, 20). Moreover, TREK-1^{-/-} mice are more sensitive (inactivation of a stretch-activated hyperpolarizing K⁺ channel) to mechanical stimuli indicating that eukaryotic MS channels can be studied at both the molecular and organismal level (1).

The experiments of Lauritzen et al show that expression of a channel protein can change cell structure regardless of whether or not the channel is permeant, so the proper control for transfection is far from obvious (15). Similar changes in background currents may be elicited by cytoskeletal disrupting agents. For example PC12 cells treated with cytochalasin-D increased the background MSC current 3 fold (2.9 ± 0.6 pS/mmHg [n=7] vs 0.9 ± 0.1 pS/mmHg [n=6], data not shown). Background MscCa are often functionally concealed, but they can be exposed by repeated stimulation (19, 21, 22). In any case, given that all cells have

background MscCa, the minimum requirement for dependable results should be a double blind experiment.

In conclusion, although several TRPC channel members, including TRPC1 and TRPC6, have been proposed to form MscCa in vertebrate cells (18, 23), the functional expression of these subunits in heterologous systems remains problematic. In particular, the variable levels of background MscCa expression seen in all mammalian cell lines, and the yet to be defined factors that regulate this expression complicate experiments comparing activity in mammalian sublines. Clearly, further studies are needed to identify the interacting and regulatory components required for the proper maturation and/or functioning of this class of ion channels. The present results are in sharp contrast with the original reports claiming that both TRPC1 and TRPC6 are components of the MscCa (18, 23). Differences in cell lines, transfection methods, trafficking, protein maturation, endogenous subunits may certainly be at play and explain these experimental discrepancies. However, in *Xenopus* oocytes, TRPC1 may reach the plasma membrane (Fig. 5d) and thus contribute to the activity of MscCa (18).

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Figure 1: Stretch-activated currents averaged across many patches in the cell-attached patch configuration in transiently transfected COS-7 cells. a) Empty expression vector (n = 16). b) TREK-1 (n = 10). c) Mock transfection with the empty expression vector (n = 70). d) hTRPC1 (n = 70). The pressure pulse protocol is shown in top panel a. Each color indicates a pressure value. The holding potential was -100 mV for panels a-b and 0 mV for panels c-d. Currents are inward in a and b and outward in d.

Fig. 2: a) Cell-attached patch single channel recordings at different pressures as indicated at a holding potential of -100 mV in a mock-transfected COS-7 cells. b) Single channel I-V curves in the presence of external 150 mM NaCl (n = 4), external choline chloride (n = 3) and external Na gluconate (n = 3) demonstrating that channels are nonselective and cationic. Currents were recorded in mock-transfected COS cells during a pressure pulse of -30 mm Hg.

Fig. 3: a) shows peak current amplitude elicited by a -80 mm Hg pressure in the cell-attached configuration measured at -100 mV. hTRPC1 expressing cells (shown in red; n = 70) and mock-transfected cells expressing the empty expression vector (pIRES2 EGFP) (shown in black ; n = 140) are illustrated. b) shows normalized frequency as a function of peak current amplitude (5 pA bins). Patches from hTRPC1 expressing cells (n = 70) are shown by red bars while control currents (n = 140) are shown in black.

Fig. 4: a) Pressure-response curves for COS cells transfected with the empty expression vector (n = 70), hTRPC1 (n = 70) or mTREK-1 (n = 10). TREK1 produces large currents unlike TRPC1. b) Expanded current scale for the mock and hTRPC1

conditions showing that the pressure dependence of the mock and TRPC1 cells are the same. Currents were recorded in the cell-attached patch configuration at a holding potential of -100 mV (mock and TRPC1) or 0 mV (TREK-1).

Figure 5: a) Confocal image of CHO cells transfected with EYFP-mTREK-1. b) Confocal image of CHO cells transfected with hTRPC1-EYFP. Both sections are at about the same height above the coverslip. c) A high magnification confocal fluorescent image focused on the edge of an oocyte that had been injected 3 days earlier with an mRNA construct encoding enhanced green fluorescence protein (eGFP) attached to the C-terminus of hTRPC1. GFP-TRPC1 is concentrated at the membrane surface of the *Xenopus* oocyte. The insert shows the same oocyte at lower magnification (1 mm in diameter). d) Expression of flag-tagged hTRPC1 in transiently transfected COS cells. Nuclei were stained with DAPI in blue and hTRPC1 expression is shown in red. Note the obvious localization of hTRPC1 in the endoplasmic reticulum.

Fig. 6: Pressure sensitive peak currents in CHO cells for patches collected over several months. Notice the log scale that suppresses the apparent scatter. Membrane potential: -90mV. hTRPC1 transfected CHO cells (red circles) and control cells (black squares). The data of Maroto et al. (18) are shown as blue asterisks for TRPC1 expressing cells and the control is shown by C.

Fig. 7: a) Expression of amino terminal HA tagged hTRPC6 in COS cells. Nuclei were stained with DAPI in blue and hTRPC6 expression is shown in red. b) Stretch-activated currents averaged across 40 patches in the cell-attached patch configuration in transiently transfected COS-7 cells with the empty expression

vector (n = 40). c) hTRPC6 (n = 41). The pressure pulse protocol is shown in panel b. d) Effect of 100 μ M OAG on channel activity of an inside-out patch expressing hTRPC6. e) Pressure sensitive peak currents in COS cells for patches collected over several transfections. hTRPC6 transfected CHO cells (blue circles) and control cells (black circles). f) Normalized frequency as a function of peak current amplitude (5 pA bins). Patches from hTRPC6 expressing cells (n = 41) are shown by blue bars while control currents (n = 40) are shown in black. In b-f, the holding potential was -100 mV and currents are inward. The pressure stimulation was -60 mm Hg.

Movie Supp 1: Movie (.avi) of 3D reconstruction of EYFP labelled TREK-1 showing localization in the plasma membrane (The DivX codec is recommended: <http://www.divx.com/divx/windows/author/?lang=en>).

Movie Supp 2: Movie (.avi) of 3D reconstruction of EYFP labelled hTRPC1 showing a presence throughout the cell but no concentration in the plasma membrane (The DivX codec is recommended: <http://www.divx.com/divx/windows/author/?lang=en>).

Materials and methods

Cell culture, plasmid construction, transfection with DEAE-dextran or Fugene, and the electrophysiological procedures have been extensively detailed elsewhere (18, 20). TREK-1 (Accession N^o: AY736359), hTRPC1 (alternatively spliced sequence accession N^o: NM003304) and hTPRPC6 (Accession N^o: NM004621) were transiently transfected in CHO or COS-7 cells. The same pIRES2 EGFP expression vector was used for functional expression of both channel types in transiently transfected COS and CHO cells. We routinely used 0.5 µg DNA per 35 mm diameter plate containing ~ 30,000 cells. Patch pipettes were ~ 1.5 MΩ. Membrane stretch (ALA HSPC-1 pressure clamp) was applied as previously described (13). Routinely, the pipette solution contained (in mM): 150 NaCl, 5 KCl, 3 MgCl₂, 1 CaCl₂, and 10 HEPES, pH 7.4 with NaOH and the bath solution contained (in mM): 155 KCl, 5 EGTA, 3 Mg²⁺, 10 HEPES at pH 7.2. Amino-terminal EYFP-tagged mTREK-1 channel and carboxy terminal EYFP-tagged hTRPC1 were used for channel localization in transfected mammalian cells. The EYFP-mTREK-1 fusion protein showed no functional difference with the WT channel. In some experiments an amino terminal Flag tagged hTRPC1 construct and an amino terminal HA tagged hTRPC6 were used. After mounting, specimens were observed using an epifluorescence microscope (Axioplan 2, Carl Zeiss) with appropriate filters. Images were recorded with a cooled CCD camera (Coolsnap HQ, Photometrics) driven by Metavue software. Three dimensional reconstructions and stereo pairs were made using a ZEISS confocal microscope and relevant software. Other methods are essentially as described by Maroto et al¹. CHO-K1 cells (ATCC, Manassa, VA) were cultured in RPMI 1640 medium

supplemented with 25 mM Hepes and glutamine, 8 % fetal calf serum, 1 mM Na pyruvate, 4.5 g/L glucose and antibiotics at 37°C in a humidified 95% O₂-5% CO₂ atmosphere. Cells were transfected with TRPC1-XOOM using Fugene HD (Roche, Indianapolis, IN) and sorted by FACS. Patch-clamp experiments were carried out after 2-5 days of seeding cells on glass cover slips. *Xenopus* oocytes were injected with mRNA transcripts of eGFP-hTRPC1 and cells were studied 3-4 days after injection.

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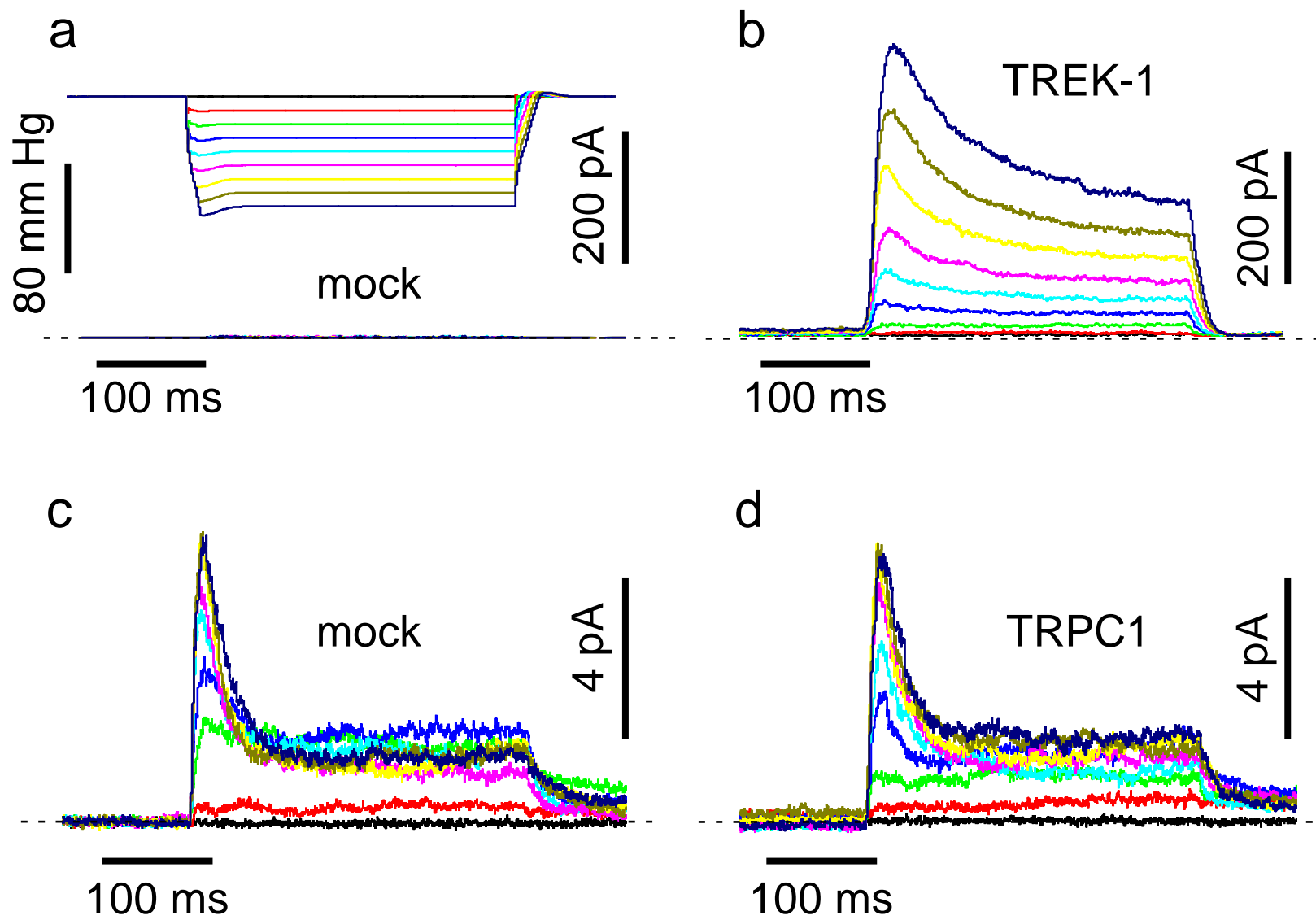
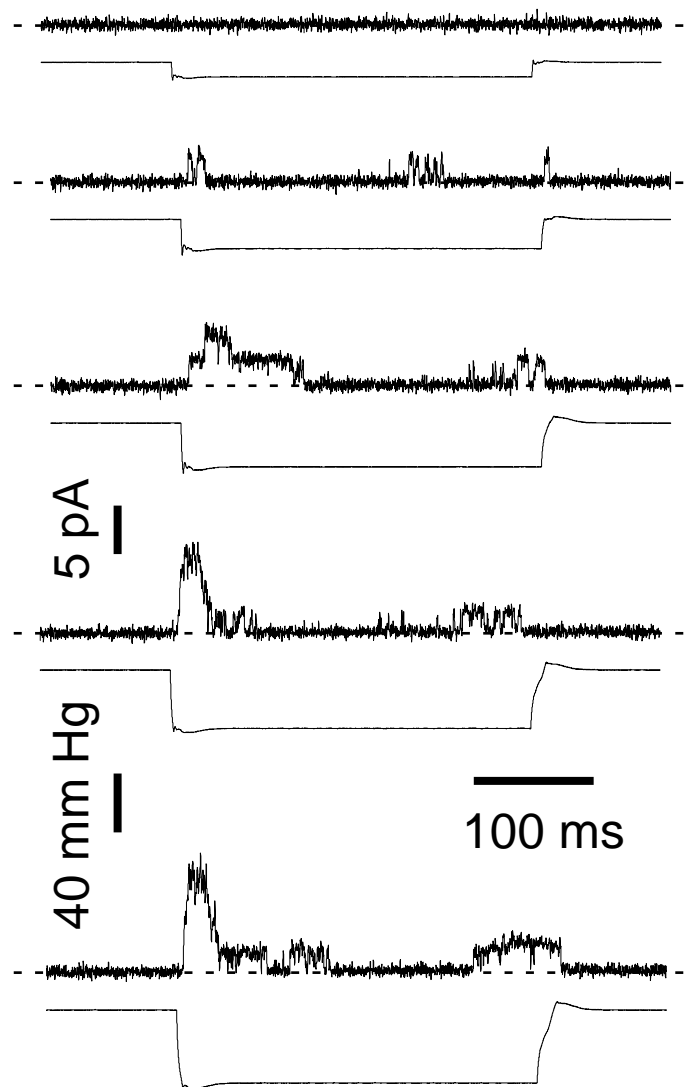


Fig. 1

a

COS cell
Cell-attached configuration @ -100 mV



b

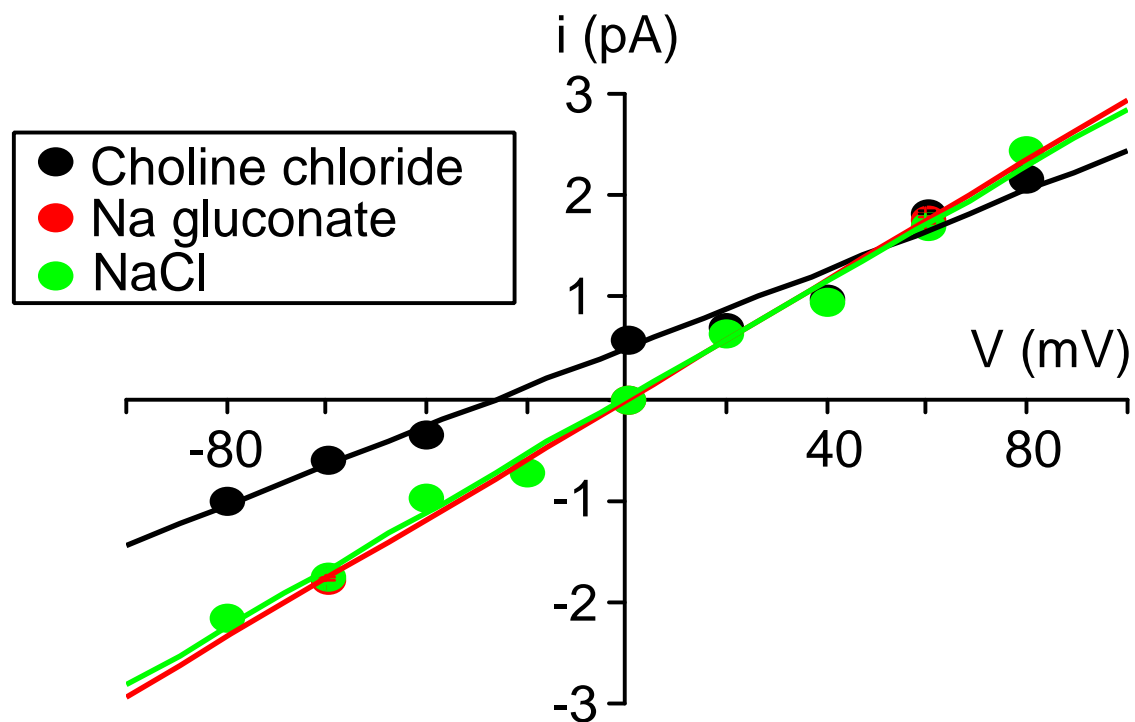


Fig. 2

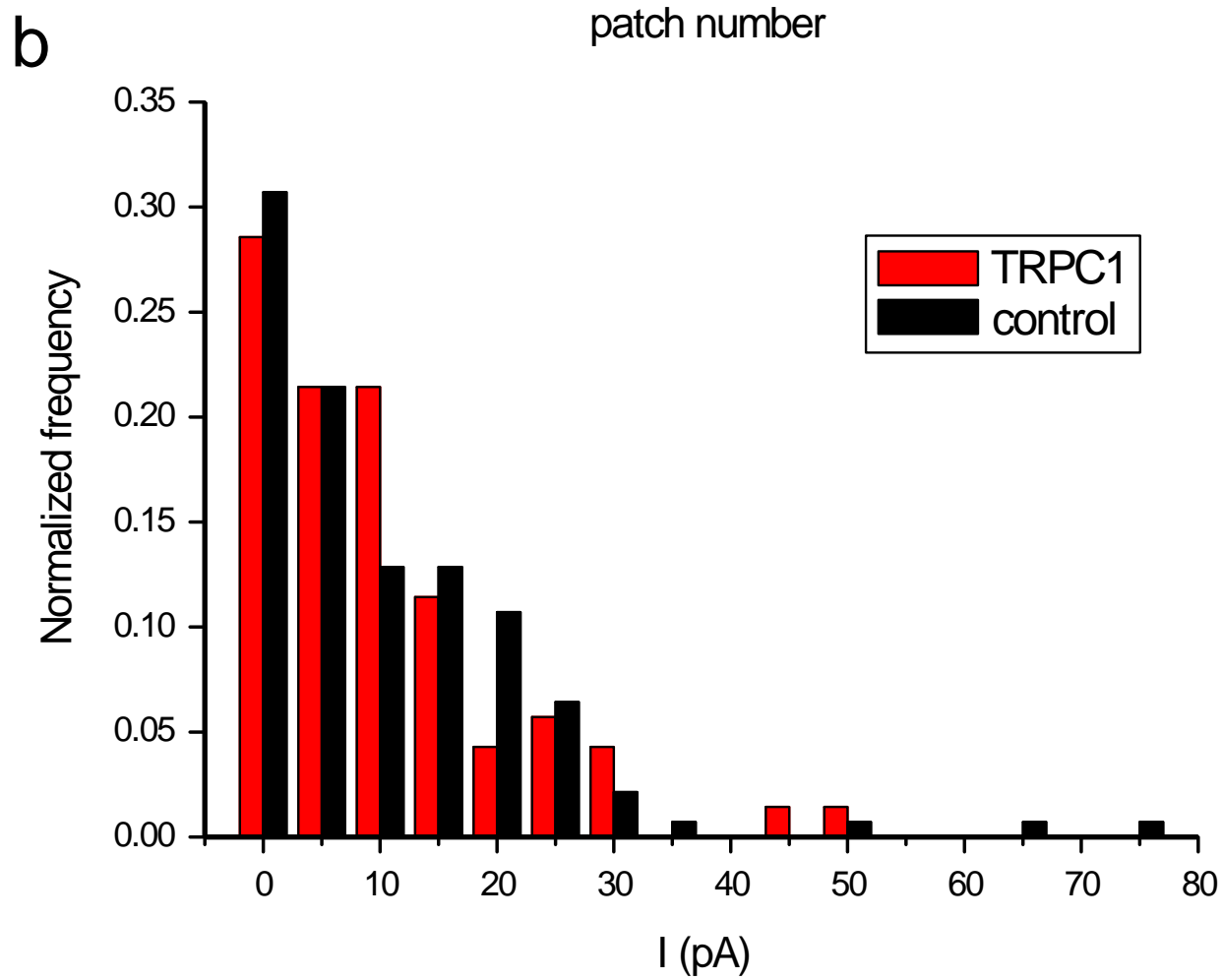
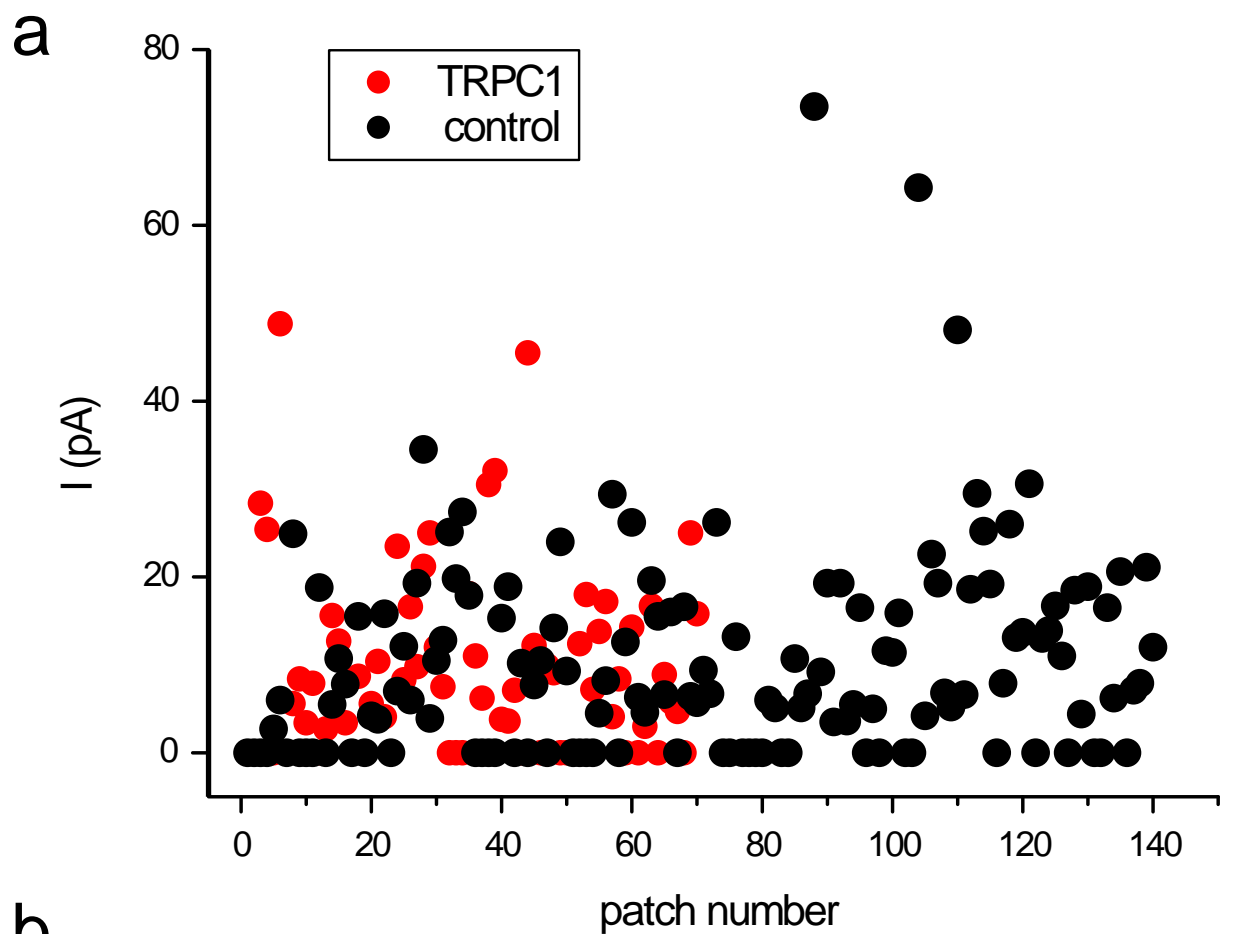


Fig. 3

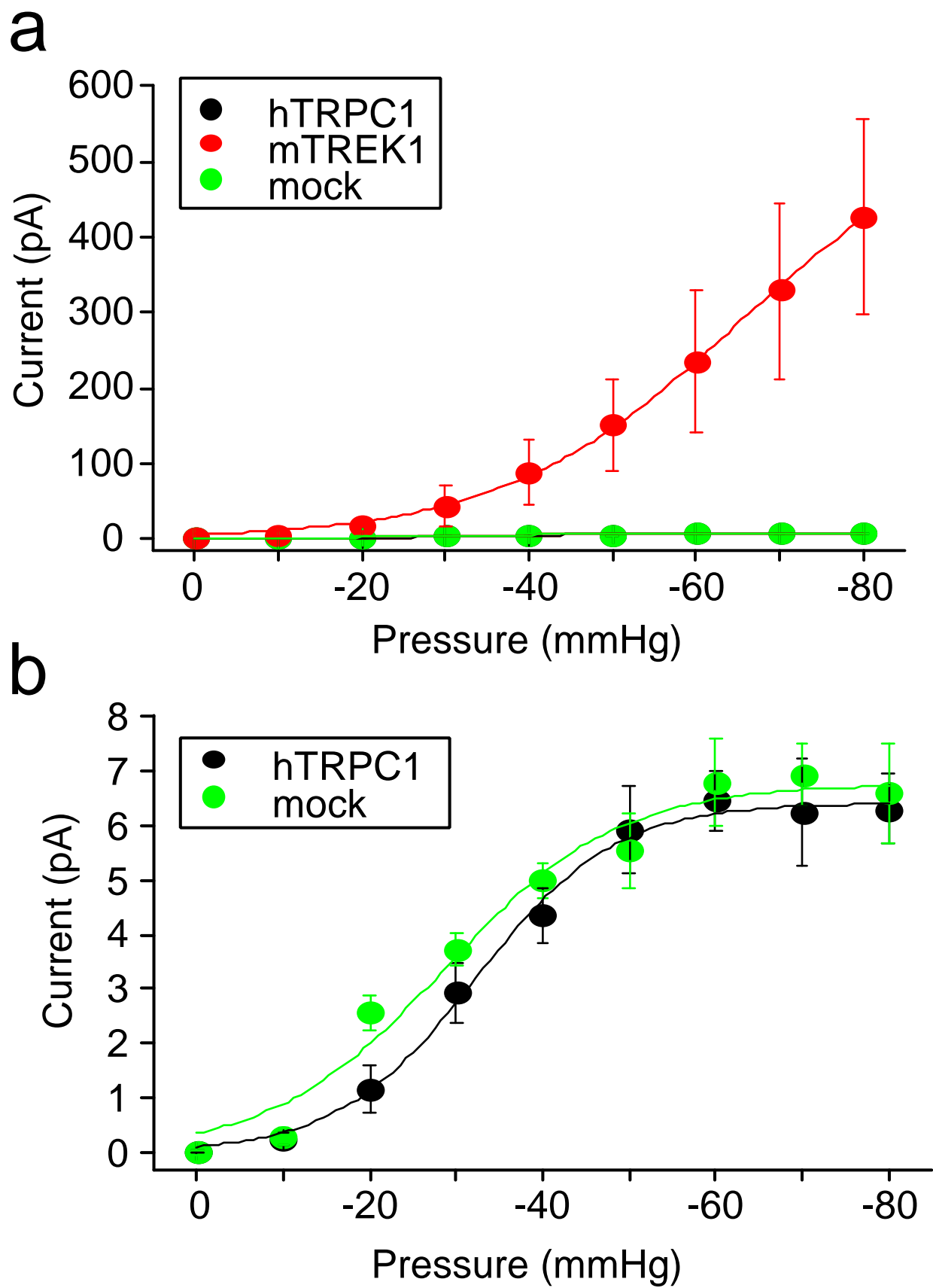


Fig. 4

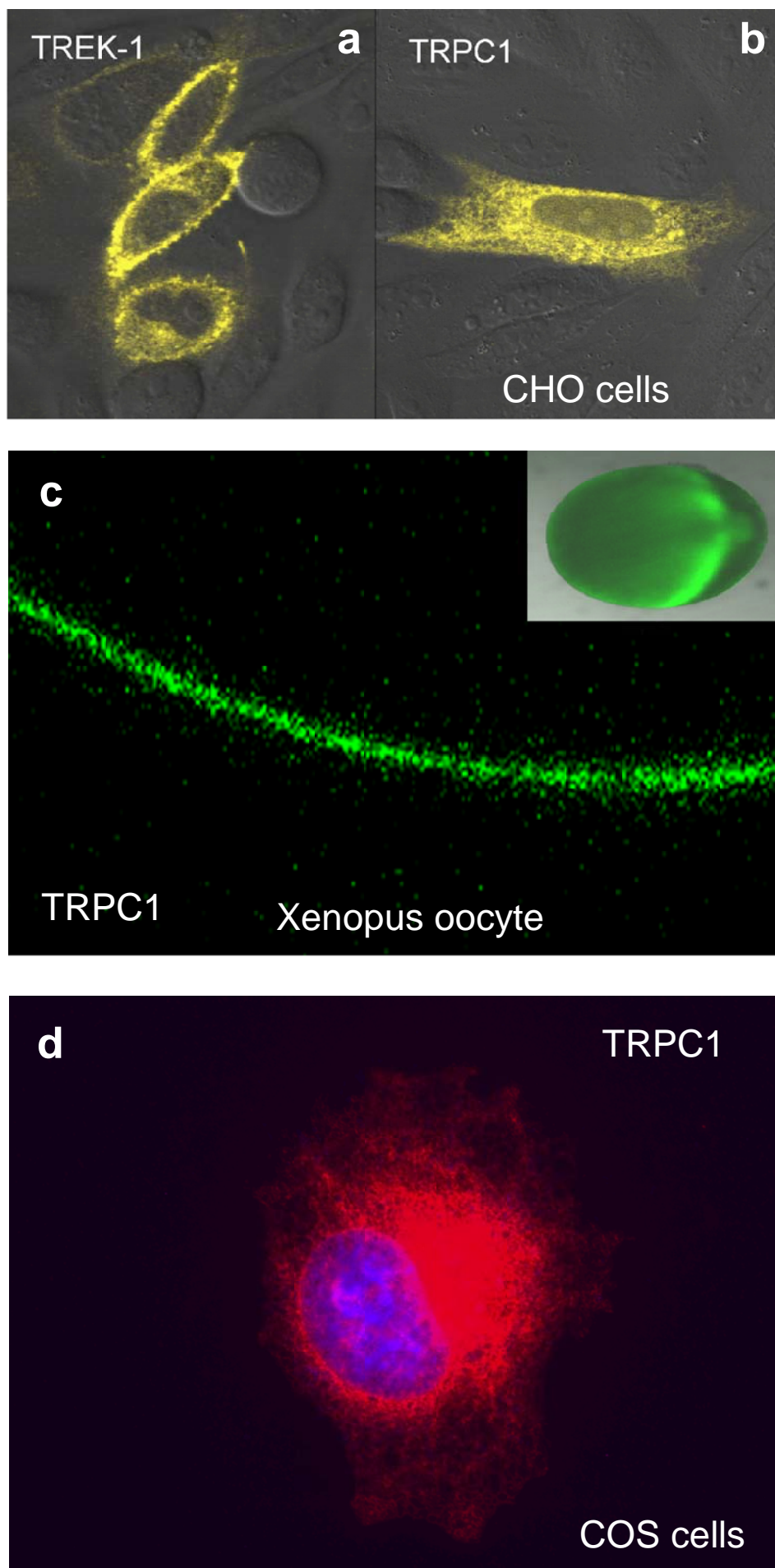


Fig. 5

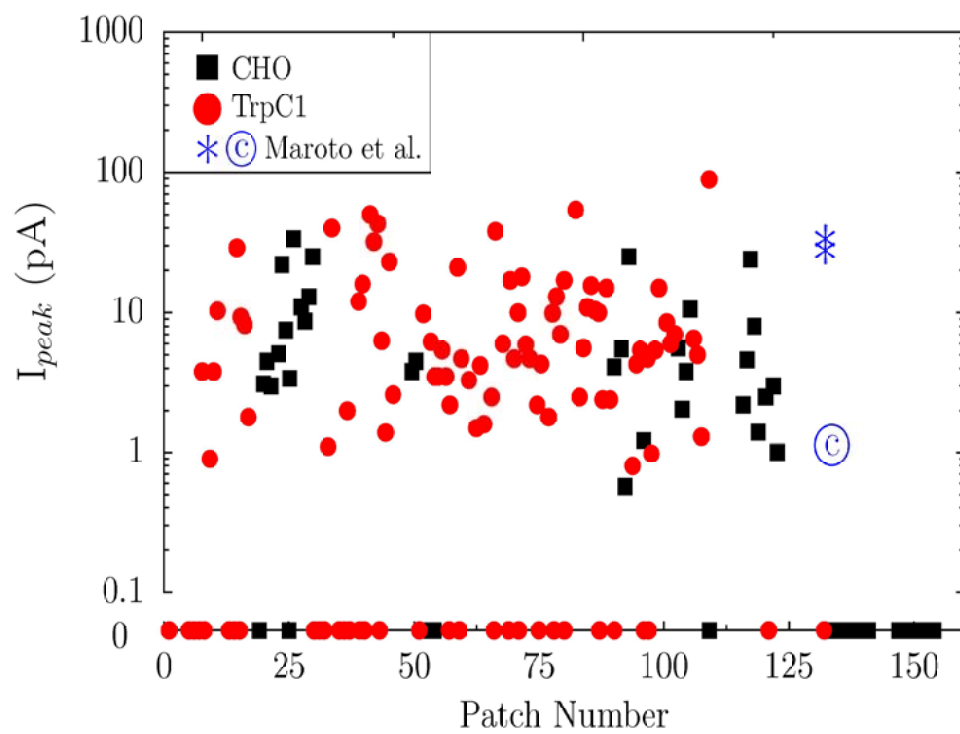


Fig. 6

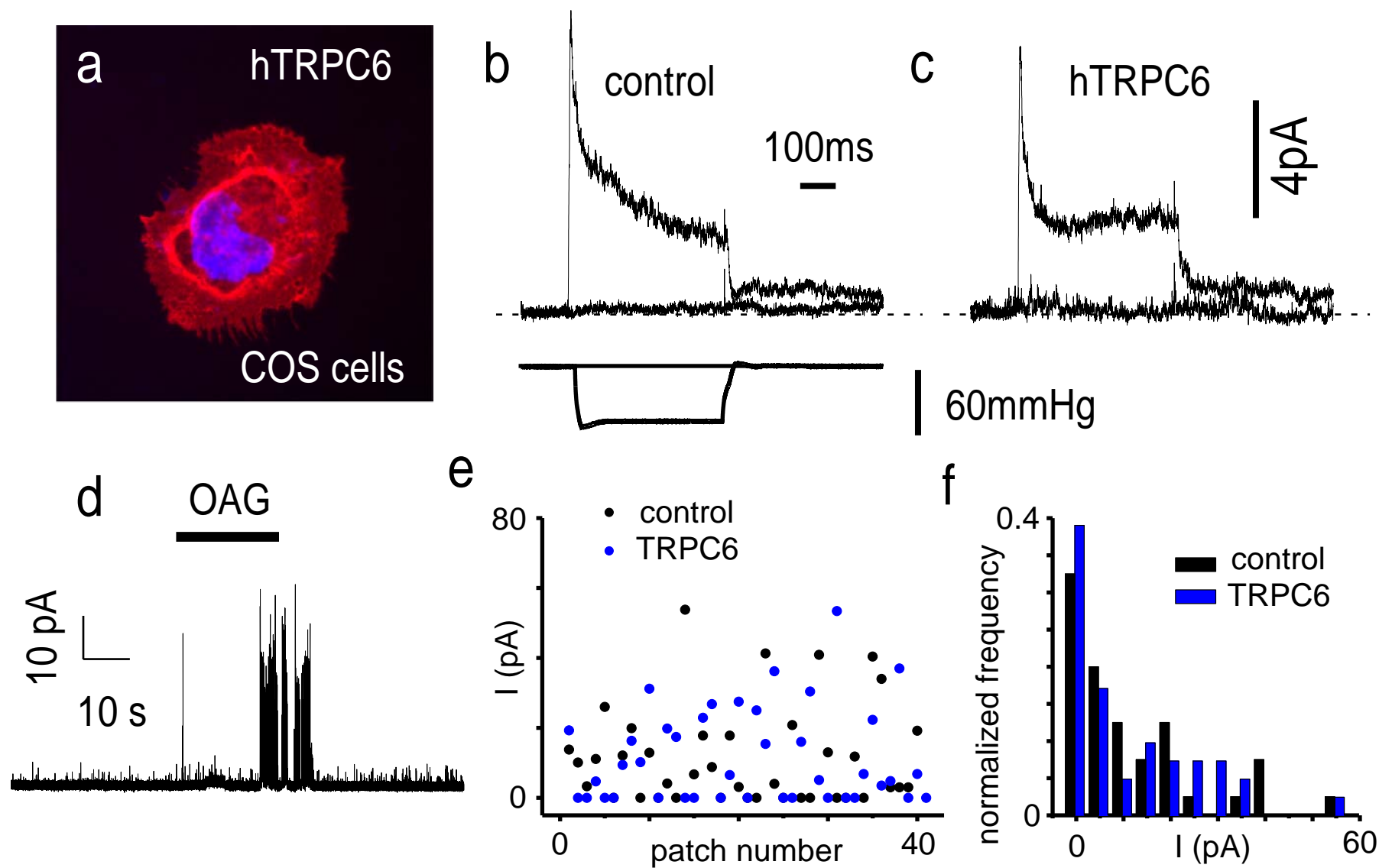


Fig. 7

Speaker Abstracts

008 • Wednesday, September 19 • TRPs: Taste and Mechanosensation • Hamill

The Role of the Mechanosensitive Ca^{2+} Permeant Channel in Prostate Tumor Cell Migration

Maroto, R., Kurosky, A. and Hamill, O.P.
University of Texas Medical Branch, Galveston TX

The mechanosensitive Ca^{2+} permeant channel (MscCa) transduces membrane stretch into Ca^{2+} influx and has been proposed to regulate fish keratocyte locomotion by coordinating forward cell extension with Ca^{2+} -sensitive mechanisms that promote rear cell retraction (Lee et al., 1999). Patch clamp recordings indicate that MscCa is also expressed by the highly motile/invasive human prostate tumor cell line PC-3, and displays the same conductance and voltage-dependent open channel gating as the *Xenopus* MscCa formed by the canonical transient receptor potential channel (TRPC1). Agents that block MscCa, including Gd^{3+} , GsMTx-4, and an anti-TRPC1 antibody, block cell migration as well as the sustained intracellular $[\text{Ca}^{2+}]_i$ gradient (front low-rear high) that determines migration directionality. Non-motile cells also express MscCa. In particular, the nonmigratory/noninvasive PC cell line LNCaP expresses a higher MscCa/TRPC1 density than PC-3 cells. However, unlike in PC-3 cells, MscCa in LNCaP cells undergoes rapid inactivation (~100 ms) in response to stretch and is therefore unable to generate the sustained $[\text{Ca}^{2+}]_i$ gradient that might otherwise support directional migration. We find that mechanical stimulation of the LNCaP cell membrane can switch MscCa gating from the transient to the sustained mode. In this case, the mechanical stresses that develop within a growing tumor may switch on tumor cell migration by modulating MscCa gating. We propose that MscCa and the mechanisms that regulate its gating mode represent promising new targets to block tumor cell migration and invasion. In particular, because Ca^{2+} influx via MscCa appears important in regulating all major modes of cell migration (i.e., mesenchymal, amoeboid and collective) it may be more effective than other more selective strategies (e.g., integrins and metalloproteinases) that fail *in vivo* as a consequence of migration mode plasticity (Wolf & Friedl, 2006; Maroto & Hamill, 2007).

Supported by the United States Army Medical Research Command (PC061444) and the National Cancer Institute (CA106629).

Lee, J., Ishihara, A., Oxford, G., Johnson, B. and Jacobson, K. (1999). Regulation of cell movement is mediated by stretch-activated calcium channels. *Nature*, 400, 382-386.

Maroto, R. and Hamill, O.P. (2007). MscCa regulation of tumor cell migration and metastasis. *Current Topics in Membranes*, 59, 485-509.

Wolf, K. and Friedl, P. (2006). Molecular mechanisms of cancer cell invasion and plasticity. *British Journal of Dermatology*, 154, 11-15.

The Stretch-activated Calcium Channel as a Central Regulator of Prostate Cancer Cell Migration

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In its early stages, prostate cancer (PC) stays in the prostate and is not life threatening, but without treatment it spreads to other parts of the body and eventually causes death. Because the acquisition of cell motility is a critical step in the metastatic cascade, it is important to identify the mechanisms that regulate PC cell migration. In order for any cell to migrate, there must be coordination between its forward protrusion and rear retraction. It has been hypothesized that the mechanosensitive stretch-activated Ca^{2+} -permeable channel (MscCa) plays a central role in this coordination by providing feedback between cell extension and the Ca^{2+} -sensitive proteins (e.g., myosin II and calpain) that promote cell contraction and/or rear adhesion disassembly. With the funding support provided by a 2004/2005 Exploration Hypothesis Development Award, we tested this hypothesis by using patch-clamp recording to measure whether MscCa is expressed in the highly motile/invasive human prostate tumor cell line PC-3, and if so, how this channel activity might influence Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) changes associated with cell migration measured using intracellular $[\text{Ca}^{2+}]_i$ imaging and time-lapse video microscopy. At the same time we also examined MscCa activity and $[\text{Ca}^{2+}]_i$ changes in the nonmotile/noninvasive PC cell line LNCaP. Our results indicate that MscCa is expressed in PC-3 cells and is essential for PC-3 cell migration. In particular, we found that several diverse agents including GD3+, the tarantula venom peptide, GsMTx-4, and an antibody raised against the pore domain of the transient receptor potential channel 1, which appear to only share the common feature of targeting MscCa (or the TRPC protein implicated in forming MscCa) blocks both the development of a sustained $[\text{Ca}^{2+}]_i$ gradient (low in the front and high in the rear) and cell migration. However, we also found that MscCa alone is not sufficient to confer motility on PC cells. Instead, our results indicate that MscCa needs to show a polarized surface distribution and express a sustained gating mode that can generate a sustained and polarized Ca^{2+} influx during cell extension in the migrating cell. For example, although the nonmotile LNCaP cells express MscCa at even higher membrane density than the motile PC-3 cells, the channels are uniformly distributed over the LNCaP cell surface and rapidly inactivate during membrane stretch. Furthermore, unlike PC-3 cells, LNCaP cells do not develop a sustained $[\text{Ca}^{2+}]_i$ gradient although they do express fast $[\text{Ca}^{2+}]_i$ transients that are also blocked by anti-MscCa agents. In conclusion our results support the original hypothesis that MscCa activity is essential for PC cell migration.

IMPACT: There is now an urgent need to identify novel and susceptible molecular mechanisms that may be targeted to block PC invasion and metastasis. Our studies indicate that MscCa and the physical/biochemical mechanisms that regulate its gating and surface distribution should be promising targets to block PC cell migration and invasion.

The U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0152 supported this work.

University of Texas/AntiCancer, Inc.

Report on the study of evaluation of the effect of anti-MscCa/TRPC agent on invasion and metastases of human prostate cancer in orthotopic MetMouse[®] model

1.0 OBJECTIVE

This study is designed to determine whether anti-MscCa/TRPC agents can block PC-3 invasion and metastases in vivo, which have shown to block prostate tumor cell migration in vitro.

2.0 INVESTIGATORS

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Robert M. Hoffman, Ph.D.

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3.0 STUDY PERIOD

This study was conducted from October 5, 2007 to November 20, 2007.

4.0 MATERIALS AND METHODS

Experimental Animals: A total of ten male, NCr nu/nu mice (5-6 weeks old) were used in the study. The experimental animals, originally purchased from Taconic (Germantown, NY), were bred and raised in AntiCancer Inc.

Cell lines: Permanently TRPC-suppressed PC-3-GFP human prostate cancer cell line and Wide-type PC-3 GFP human prostate cancer cell line were provided by the Sponsor. Two cell lines were cultured in AntiCancer, Inc.

Subcutaneous tumor stock: Tumor stock for TRPC-suppressed PC-3-GFP cell line was made by subcutaneously injecting the cells at a concentration of 5×10^6 cells

/100 µl into the right flanks of five mice on October 5, 2007. Tumor stock for Wide-type PC-3 GFP cell line was made by subcutaneously injecting the cells at a concentration of 5×10^6 cells /100 µl into the right flanks of five mice on October 8, 2007.

5.0 RESULTS

Pending